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Page 2 de l'attestation

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Bezeichnung der Erfindung: Title of the invention: Titre de l'invention:
A novel MPV belonging to the sub-family Pneumovirinae, causing respiratory tract illness in humans

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Title: A virus causing respiratory tract illness in susceptible mammals.

The invention relates to the field of virology.

In the past decades several etiological agents of mammalian disease, in particular of respiratory tract illnesses (RTI), in particular of humans, have been identified[7]. Classical etiological agents of RTI with mammals are respiratory syncytial viruses belonging to the genus *Pneumovirus* found with humans (hRSV) and ruminants such as cattle or sheep (bRSV and/or oRSV). Another classical *Pneumovirus* is the pneumonia virus of mice (PMV), in general only found with laboratory mice. However, a proportion of the illnesses observed among mammals can still not be attributed to known pathogens.

The invention provides a novel etiological agent, an isolated essentially mammalian negative-sense single stranded RNA virus (herein also called MPV) belonging to the subfamily *Pneumovirinae* of the family *Paramyxoviridae* but not identifiable as a classical pneumovirus, and most closely related to and likely belonging to the genus *Metapneumovirus*..

Mammalian viruses resembling metapneumoviruses, i.e. metapneumoviruses isolatable from mammals that essentially function as natural host for said virus or cause disease in said mammals, have until now not been found. Metapneumoviruses, in general thought to be essentially restricted to poultry as natural host or aetiological agent of disease, are also known as avian pneumoviruses. The invention provides an isolated mammalian pneumovirus comprising a gene order and amino acid sequence distinct from that of the genus *Pneumovirus* and which is closely related and considering its phylogenetic relatedness likely belonging to the genus *Metapneumovirus* within the subfamily *Pneumovirinae* of the family *Paramyxoviridae*. Although until now, all metapneumoviruses have been isolated from birds, it is now shown that closely related viruses can also be identified in other animal species such as mammals.

Since MPV CPE was virtually indistinguishable from that caused by hRSV or hPIV in tMK or other cell cultures, the MPV may have well gone unnoticed until now. The CPE is, as well as with some of the classical *Paramyxoviridae*,

characterized by syncytium formation after which the cells showed rapid internal disruption, followed by detachment of the cells from the monolayer. The cells usually (but not always) displayed CPE after three passages of virus from original material, at day 10 to 14 post inoculation, somewhat later than CPE caused by other viruses such as hRSV or hPIV.

Classically, as devastating agents of disease, paramyxoviruses account for many animal and human deaths worldwide each year. The *Paramyxoviridae* form a family within the order of *Mononegavirales* (negative-sense single stranded RNA viruses), consisting of the sub-families *Paramyxovirinae* and *Pneumovirinae*. The latter sub-family is at present taxonomically divided in the genera *Pneumovirus* and *Metapneumovirus* [1]. Human respiratory syncytial virus (hRSV), the type species of the *Pneumovirus* genus, is the single most important cause of lower respiratory tract infections during infancy and early childhood worldwide[2]. Other members of the *Pneumovirus* genus include the bovine and ovine respiratory syncytial viruses and pneumonia virus of mice (PVM).

Avian pneumovirus (APV) also known as turkey rhinotracheitis virus (TRTV), the aetiological agent of avian rhinotracheitis, an upper respiratory tract infection of turkeys[3], is the sole member of the recently assigned *Metapneumovirus* genus, which, as said was until now not associated with infections, or what is more, with disease of mammals.

In a preferred embodiment, the invention provides an isolated MPV taxonomically corresponding to a (hereto unknown mammalian) metapneumovirus comprising a gene order distinct from that of the pneumoviruses within the sub-familie *Pneumovirinae* of the family *Paramyxoviridae*. The classification of the two genera is based primarily on their gene constellation; metapneumoviruses generally lack non-structural proteins such NS1 or NS2 and the gene order is different from that of pneumoviruses (RSV: '3-NS1-NS2-N-P-M-SH-G-F-M2-L-5', APV: '3-N-P-M-F-M2-SH-G-L-5') [4,5,6]. MPV as provided by the invention or a virus isolate taxonomically corresponding therewith is upon EM analysis revealed by paramyxovirus-like particles. Consistent with the classification, MPV or virus isolates phylogenetically corresponding or taxonomically corresponding therewith are sensitive to treatment with chloroform; are cultured optimally on tMK cells or cells functionally equivalent thereto and are essentially trypsine dependent in most cell cultures. Furthermore, the typical CPE and lack of haemagglutinating

activity with most classically used red blood cells suggested that a virus as provided herein is, albeit only distantly, related to classical pneumoviruses such as RSV. Although most paramyxoviruses have haemagglutinating acitivity, most of the pneumoviruses do not [13].

To find further viral isolates as provided by the invention it suffices to test a sample, optionally obtained from a diseased animal or human, for the presence of a virus of the sub-family *Pneumovirinae*, and test a thus obtained virus for the presence of genes encoding (functional) NS1 or NS2 or essentially demonstrate a gene order that is different from that of pneumoviruses such as RSV as already discussed above. Furthermore, a virus isolate phylogenetically corresponding and thus taxonomically corresponding with MPV may be found by cross-hybridisation experiments using nucleic acid from a here provided MPV isolate, or in classical cross-serology experiments using monoclonal antibodies specifically directed against and/or antigens and/or immunogens specifically derived from an MPV isolate.

Newly isolated viruses are phylogenetically corresponding to and thus taxonomically corresponding to MPV when comprising a gene order and/or amino acid sequence sufficiently similar to our prototypic MPV isolate(s), or are structurally corresponding therewith, and show close relatedness to the genus *Metapneumovirus* within the subfamily *Pneumovirinae*. The highest amino sequence homology, and defining the structural correspondence on the individual protein level, between MPV and any of the known other viruses of the same family to date (APV subtype C) is for matrix 87%, for nucleoprotein 52%, for phosphoprotein 67%, for fusionprotein 80% and for parts of the polymerase protein 56%. Individual proteins or whole virus isolates with, respectively, higher homology to these mentioned maximum values are considered phylogenetically corresponding and thus taxonomically corresponding to MPV, and comprise a nucleic acid sequence structurally corresponding with a sequence as shown in figure 6a, 6b, 6c. Herewith the invention provides a virus phylogenetically corresponding to the deposited virus.

It should be noted that, similar to other viruses, a certain degree of variation is found between different MPV isolates. In phylogenetic trees, we have identified at least 2 genetic clusters of virus isolates based on sequence analyses of L, M, N and F genes. Based on nucleotide and amino-acid differences, and in analogy to other pneumoviruses such as RSV, these MPV genotypes represent subtypes of

MPV. Within each of the genetic clusters of MPV isolates, the percentage identity at the nucleotide level was found to be 94-100 for L, 91-100 for M, 90-100 for N and 93-100 for F and at the amino acid level the percentage identity was found to be 91-100 for L, 98-100 for M, 96-100 for N and 98-100 for F. The minimum percentage identity at the nucleotide level for the entire group of MPV isolates identified so far was 81 for L and M, 83 for N and 82 for F. At the amino acid level, this percentage was 91 for L and N, 94 for M, and 95 for F. Sequence divergence of MPV strains around the world may be somewhat higher, in analogy with other viruses.

From these data we conclude that virus isolates displaying percentage amino acid homology higher than 56 for L, 87 for M, 52 for N, 67 for P or 80 for F may definitively be classified as MPV. When the percentage amino acid sequence homology for a given virus isolate is higher than 90 for L and N, 93 for M, or 94 for F, the virus isolate is similar to the group of virus isolates displayed in figure 5. When the percentage amino acid sequence homology for a given virus isolate is higher than 94 for L, 95 for N or 97 for M and F the virus isolate can be identified to belong to one of the genotype clusters represented in figure 5. It should be noted that these percentages of homology, by which genetic clusters are defined, are similar to the degree of homology found among genetic clusters in the corresponding genes of RSV.

New MPV isolates are for thus example identified by virus isolation and characterisation on tMK or other cells, by RT-PCR and/or sequence analysis followed by phylogenetic tree analyses, and by serologic techniques such as virus neutralisation assays, indirect immunofluorescence assays, direct immunofluorescence assays, FACs analyses or other immunological techniques.

For example the invention provides herein a method to identify further isolates of MPV as provided herein, the method comprising inoculating a essentially MPV-uninfected or specific-pathogen-free guinea pig or ferret (in the detailed description the animal is inoculated intranasally but other ways of inoculation such as intramuscular or intradermal inoculation, and using an other experimental animal, is also feasible) with the prototype isolate I-2614 or related isolates. Sera are collected from the animal at day zero, two weeks and three weeks post inoculation. The animal specifically seroconverted as measured in virus neutralisation (VN) assay and indirect IFA against the respective isolate

I-2614 and the sera from the seroconverted animal are used in the immunological detection of said further isolates.

As an example, the invention provides the characterisation of a new member in the family of *Paramyxoviridae*, a human metapneumovirus or metapneumovirus-like virus (since its final taxonomy awaits discussion by a viral taxonomy committee the MPV is herein for example described as taxonomically corresponding to APV) (hMPV) which may cause severe RTI in humans. The clinical signs of the disease caused by hMPV are essentially similar to those caused by hRSV, such as cough, myalgia, vomiting, fever, broncheolitis or pneumonia, possible conjunctivitis, or combinations thereof. As is seen with hRSV infected children, especially very young children may require hospitalisation. As an example an MPV which was deposited January 19, 2001 as I-2614 with CNCM, Institute Pasteur, Paris or a virus isolate phylogenetically corresponding therewith is herewith provided. Therewith, the invention provides a virus comprising a nucleic acid or functional fragment phylogenetically corresponding to a nucleic acid sequence shown in figure 6a, 6b, 6c, or structurally corresponding therewith. In particular the invention provides a virus characterised in that after testing it in phylogenetic tree analyses wherein maximum likelihood trees are generated using 100 bootstraps and 3 jumbles it is found to be more closely phylogenetically corresponding to a virus isolate deposited as I-xxxx with CNCM, Paris than it is related to a virus isolate of avian pneumovirus (APV) also known as turkey rhinotracheitis virus (TRTV), the aetiological agent of avian rhinotracheitis.

We propose the new human virus to be named human metapneumovirus or metapneumovirus-like virus (hMPV) based on several observations. EM analysis revealed paramyxovirus-like particles. Consistent with the classification, hMPV appeared to be sensitive to treatment with chloroform. HMPV is cultured optimal on tMK cells and is trypsine dependent. The clinical symptoms caused by hMPV as well as the typical CPE and lack of haemagglutinating activity suggested that this virus is closely related to hRSV. Although most paramyxoviruses have haemaglutinating acitivity, most of the pneumoviruses do not [13]

As an example, the invention provides a not previously identified paramyxovirus from nasopharyngeal aspirate samples taken from 28 children suffering from severe RTI. The clinical symptoms of these children were largely

similar to those caused by hRSV. Twenty-seven of the patients were children below the age of five years and half of these were between 1 and 12 months old. The other patient was 18 years old. All individuals suffered from upper RTI, with symptoms ranging from cough, myalgia, vomiting and fever to broncheolitis and severe pneumonia. The majority of these patients were hospitalised for one to two weeks.

The virus isolates from these patients had the paramyxovirus morphology in negative contrast electron microscopy but did not react with specific antisera against known human and animal paramyxoviruses. They were all closely related to one another as determined by indirect immunofluorescence assays (IFA) with sera raised against two of the isolates. Sequence analyses of nine of these isolates revealed that the virus is somewhat related to APV. Based on virological data, sequence homology as well as the genomic organisation we propose that the virus is a member of *Metapneumovirus* genus. Serological surveys showed that this virus is a relatively common pathogen since the seroprevalence in the Netherlands approaches 100% of humans by the age of five years. Moreover, the seroprevelance was found to be equally high in sera collected from humans in 1958, indicating this virus has been circulating in the human population for more than 40 years. The identification of this proposed new member of the *Metapneumovirus* genus now also provides for the development of means and methods for diagnostic assays or test kits and vaccines or serum or antibody compositions for viral respiratory tract infections, and for methods to test or screen for antiviral agents useful in the treatment of MPV infections.

To this extend, the invention provides among others an isolated or recombinant nucleic acid or virus-specific functional fragment thereof obtainable from a virus according to the invention. In particular, the invention provides primers and/or probes suitable for identifying an MPV nucleic acid. Furthermore, the invention provides a vector comprising a nucleic acid according to the invention. To begin with, vectors such as plasmid vectors containing (parts of) the genome of MPV, virus vectors containing (parts of) the genome of MPV. (For example, but not limited to other paramyxoviruses, vaccinia virus, retroviruses, baculovirus), or MPV containing (parts of) the genome of other viruse or other pathogens are provided. Furthermore, a number of techniques have been described for the generation of recombinant negative

strand viruses, based on two critical parameters. First, the production of such virus relies on the replication of a partial or full-length copy of the negative sense viral RNA (vRNA) genome or a complementary copy thereof (cRNA). This vRNA or cRNA can be isolated from infectious virus, produced upon in-vitro transcription, or produced in cells upon transfection of nucleic acids. Second, the production of recombinant negative strand virus relies on a functional polymerase complex. Typically, the polymerase complex of pneumoviruses consists of N, P, L and evt. M2 proteins, but is not necessarily limited thereto. Polymerase complexes or components thereof can be isolated from virus particles, isolated from cells expressing one or more of the components, or produced upon transfection of specific expression vectors.

Infectious copies of MPV can be obtained when the above mentioned vRNA, cRNA, or vectors expressing these RNAs are replicated by the above mentioned polymerase complex [16,17,18,19,20,21,22].

Also, the invention provides a host cell comprising a nucleic acid or a vector according to the invention. Plasmid or viral vectors containing the polymerase components of MPV (presumably N, P, L and M2, but not necessarily limited thereto) are generated in prokaryotic cells for the expression of the components in relevant cell types (bacteria, insect cells, eukaryotic cells). Plasmid or viral vectors containing full-length or partial copies of the MPV genome will be generated in prokaryotic cells for the expression of viral nucleic acids in-vitro or in-vivo. The latter vectors may contain other viral sequences for the generation of chimeric viruses or chimeric virus proteins, may lack parts of the viral genome for the generation of replication defective virus, and may contain mutations, deletions or insertions for the generation of attenuated viruses.

Infectious copies of MPV (being wild type, attenuated, replication-defective or chimeric) can be produced upon co-expression of the polymerase components according to the state-of-the-art technologies described above.

In addition, eukaryotic cells, transiently or stably expressing one or more full-length or partial MPV proteins can be used. Such cells can be made by transfection (proteins or nucleic acid vectors), infection (viral vectors) or transduction (viral vectors) and may be useful for complementation of mentioned wild type, attenuated, replication-defective or chimeric viruses.

A chimeric virus may be of particular use for the generation of recombinant vaccines protecting against two or more viruses [23,24,26]. For

example, it can be envisaged that a MPV virus vector expressing one or more proteins of RSV or a RSV vector expressing one or more proteins of MPV will protect individuals vaccinated with such vector against both virus infections. Attenuated and replication-defective viruses may be of use for vaccination purposes with live vaccines as has been suggested for other viruses [25,26].

In a preferred embodiment, the invention provides a proteinaceous molecule or metapneumovirus-specific viral protein or functional fragment thereof encoded by a nucleic acid according to the invention. Useful proteinaceous molecules are for example derived from any of the genes or genomic fragments derivable from a virus according to the invention. Such molecules, or antigenic fragments thereof, as provided herein, are for example useful in diagnostic methods or kits and in pharmaceutical compositions such as sub-unit vaccines. Particularly useful are the F and/or G protein or antigenic fragments thereof for inclusion as antigen or subunit immunogen, burt inactivated whole virus can also be used.

Also provided herein are antibodies, be it natural polyclonal or monoclonal, or synthetic (e.g. (phage) library-derived binding molecules) antibodies that specifically react with an antigen comprising a proteinaceous molecule or MPV-specific functional fragment thereof according to the invention. Such antibodies are useful in a method for identifying a viral isolate as an MPV comprising reacting said viral isolate or a component thereof with an antibody as provided herein. This can for example be achieved by using purified or non-purified MPV or parts thereof (proteins, peptides) using ELISA, RIA, FACS or similar formats of antigen detection assays (*Current Protocols in Immunology*). Alternatively, infected cells or cell cultures may be used to identify viral antigens using classical immunofluorescence or immunohistochemical techniques.

Other methods for identifying a viral isolate as a MPV comprise reacting said viral isolate or a component thereof with a virus specific nucleic acid according to the invention, in particular where said mammalian virus comprises a human virus.

In this way the invention provides a viral isolate identifiable with a method according to the invention as a mammalian virus taxonomically corresponding to a negative-sense single stranded RNA virus identifiable as likely belonging to the genus *Metapneumovirus* within the sub-familie *Pneumovirinae* of the family *Paramyxoviridae*.

The method is useful in a method for virologically diagnosing an MPV infection of a mammal, said method for example comprising determining in a sample of said mammal the presence of a viral isolate or component thereof by reacting said sample with a nucleic acid or an antibody according to the invention. Examples are further given in the detailed description, such as the use of PCR (or other amplification or hybridisation techniques well known in the art) or the use of immunofluorescence detection (or other immunological techniques known in the art)

The invention also provides a method for serologically diagnosing a MPV infection of a mammal comprising determining in a sample of said mammal the presence of an antibody specially directed against a MPV or component thereof by reacting said sample with a proteinaceous molecule or fragment thereof or an antigen according to the invention.

Methods and means provided herein are particularly useful in a diagnostic kit for diagnosing a MPV infection, be it by virological or serological diagnosis. Such kits or assays may for example comprise a virus, a nucleic acid, a proteinaceous molecule or fragment thereof, an antigen and/or an antibody according to the invention.

Use of a virus, a nucleic acid, a proteinaceous molecule or fragment thereof, an antigen and/or an antibody according to the invention is also provided for the production of a pharmaceutical composition, for example for the treatment or prevention of MPV infections and/or for the treatment or prevention of respiratory tract illnesses, in particular in humans. Attenuation of the virus can be achieved by established methods developed for this purpose, including but not limited to the use of related viruses of other species, serial passages through laboratory animals or/and tissue/cell cultures, site directed mutagenesis of molecular clones and exchange of genes or gene fragments between related viruses.

A pharmaceutical composition comprising a virus, a nucleic acid, a proteinaceous molecule or fragment thereof, an antigen and/or an antibody according to the invention can for example be used in a method for the treatment or prevention of a MPV infection and/or a respiratory illness comprising providing an individual with a pharmaceutical composition according to the invention. This is most useful when said individual comprises a human, especially when said human is below 5 years of age, since such infants and young

children are most likely to be infected by a human MPV as provided herein. Generally, in the acute phase patients will suffer from upper respiratory symptoms predisposing for other respiratory and other diseases. Also lower respiratory illnesses may occur, predisposing for more and other serious conditions.

The invention also provides method to obtain an antiviral agent useful in the treatment of respiratory tract illness comprising establishing a cell culture or experimental animal comprising a virus according to any one of claims 1 to 7, treating said culture or animal with an candidate antiviral agent, and determining the effect of said agent on said virus or its infection of said culture or animal. An example of such an antiviral agent comprises a MPV-neutralising antibody, or functional component thereof, as provided herein, but antiviral agents of other nature are obtained as well. The invention also provides use of an antiviral agent according to the invention for the preparation of a pharmaceutical composition, in particular for the preparation of a pharmaceutical composition for the treatment of respiratory tract illness, especially when caused by an MPV infection, and provides a pharmaceutical composition comprising an antiviral agent according to the invention, useful in a method for the treatment or prevention of an MPV infection or respiratory illness, said method comprising providing an individual with such a pharmaceutical composition.

The invention is further explained in the detailed description without limiting it thereto.

Figure legends

Figure 1A comprises table 1: Percentage homology found between the putative amino acid sequence of isolate 00-1 and other members of the Pneumovirinae. Percentages (x100) are given for the putative amino acid sequences of N, P, M, F and two RAP-PCR fragments in L (8 and 9/10). Accession numbers used for the analyses are described in the materials and methods section.

Fig 1B comprises table 2: Seroprevalence of hMPV in humans categorised by age group using immunofluorescence and virus neutralisation assays..

Fig. 2: Schematic representation of the genome of APV with the location and size of the fragments obtained with RAP-PCR and RT-PCR on virus isolate 00-1. Fragments 1 to 10 were obtained using RAP-PCR. Fragment A was obtained with a primer in RAP-PCR fragment 1 and 2 and a primer designed based on alignment of leader and trailer sequences of APV and RSV[6]. Fragment B was obtained using primers designed in RAP-PCR fragment 1 and 2 and RAP-PCR fragment 3. Fragment C was obtained with primers designed in RAP-PCR fragment 3 and RAP-PCR fragment 4,5,6 and 7.

For all phylogenetic trees, (figures 3-5) DNA sequences were aligned using the ClustalW software package and maximum likelihood trees were generated using the DNA-ML software package of the Phylip 3.5 program using 100 bootstraps and 3 jumbles[15]. Previously published sequences that were used for the generation of phylogenetic trees are available from Genbank under accessions numbers : For all ORFs: hRSV: NC001781; bRSV: NC001989; For the F ORF: PVM, D11128; APV-A, D00850; APV-B, Y14292; APV-C, AF187152; For the N ORF: PVM, D10331; APV-A, U39295; APV-B, U39296; APV-C, AF176590; For the M ORF: PMV,U66893; APV-A, X58639; APV-B, U37586; APV-C, AF262571; For the P ORF: PVM, 09649; APV-A, U22110, APV-C, AF176591. Phylogenetic analyses for the nine different virus isolates of MPV were performed with APV strain C as outgroup.

Abbreviations used in figures: hRSV: human RSV; bRSV: bovine RSV; PVM: pneumonia virus of mice; APV-A,B,and C: avian pneumovirus typa A, B and C.

Fig. 3 Comparison of the N, P, M and F ORF's of members of the subfamily *Pneumovirinae* and virus isolate 00-1. The alignment shows the putative amino acid sequence of the complete N, P, M and F proteins and partial L proteins of virus isolate 00-1. Amino acids that differ between isolate 00-1 and the other viruses are shown, identical amino acids are represented by periods, gaps are represented as dashes. Numbers correspond to amino acid positions in the proteins. Accession numbers used for the analyses are described in the materials and methods section. APV-A, B or C: Avian Pneumovirus type A, B or C, b-or hRSV: bovine or human respiratory syncytial virus, PVM: pneumonia virus of mice. L8: fragment 8 obtained with RAP-PCR located in L, L9/10: consensus of fragment 9 and 10 obtained with RAP-PCR, located in L. For the P allignment, no APV-B sequence was available from the Genebank, For the L allignment only bRSV, hRSV and APV-A sequences were available.

Fig. 4: Phylogenetic analyses of the N, P, M, and F ORF's of members of the genus Pneumovirinae and virus isolate 00-1. Phylogenetic analysis was performed on viral sequences from the following genes: F (panel A), N (panel B), M (panel C), and P (panel D). The phylogenetic trees are based on maximum likelyhood analyses using 100 bootstraps and 3 jumbles. The scale representing the number of nucleotide changes is shown for each tree.

Fig. 5: Phylogenetic relationship for parts of the F (panel A), N (panel B), M (panel C) and L (panel D) ORFs of nine of the primary MPV isolates with APV-C, it's closest relative genetically. The phylogenetic trees are based on maximum likelyhood analyses. The scale representing the number of nucleotide changes is shown for each tree. Accesion numbers for APV-C: panel A: D00850; panel B: U39295; panel C: X58639; and panel D: U65312.

Fig. 6A: Nucleotide and amino acid sequence information from the 3'end of the genome of MPV isolate 00-1.ORF's are given. N: ORF for nucleoprotein; P: ORF for phosphoprotein; M: ORF for matrix protein; F: ORF for fusion protein; GE: putative gene end; GS: putative gene start.

Fig. 6B and C: Nucleotide and amino acid sequence information from obtained fragments in the polymerase gene (L) of MPV isolates 00-1. Positioning of the fragments in L is based on protein homologies with APV-C (accession number U65312). The translated fragment 8 (Fig. 6B.) is located at amino acid number 8 to 243, and the consensus of fragments 9 and 10 (Fig. 6C) is located at amino acid number 1358 tc 1464 of the APV-C L ORF.

Detailed description

Virus isolation and characterisation

From 1980 till 2000 we found 28 unidentified virus isolates from patients with severe respiratory disease. These 28 unidentified virus isolates grew slowly in tMK cells, poorly in VERO cells and A549 cells and could not or only little be propagated in MDCK or chicken embryonated fibroblast cells. Most of these virus isolates induced CPE after three passages on tMK cells, between day ten and fourteen. The CPE was virtually indistinguishable from that caused by hRSV or hPIV in tMK or other cell cultures, characterised by syncytium formation after which the cells showed rapid internal disruption, followed by detachment of the cells from the monolayer. The cells usually (sometimes later) displayed CPE after three passages of virus from original material, at day 10 to 14 post inoculation, somewhat later than CPE caused by other viruses such as hRSV or hPIV.

We used the supernatants of infected tMK cells for EM analysis which revealed the presence of paramyxovirus-like virus particles ranging from 150 to 600 nanometer, with short envelope projections ranging from 13 to 17 nanaometer. Consistent with the biochemical properties of enveloped viruses such as the *Paramyxoviridae*, standard chloroform or ether treatment[8] resulted in $>10^4$ TCID50 reduction of infectivity for tMK cells. Virus-infected tMK cell culture supernatants did not display heamagglutinating activity with turkey, chicken and guinea pig erythrocytes. During culture, the virus replication appeared to be trypsine dependent on the cells tested. These combined virological data allowed that the newly identified virus was taxonomically classified as a member of the *Paramyxoviridae* family.

We isolated RNA from tMK cells infected with 15 of the unidentified virus isolates for reverse transcription and polymerase chain reaction (RT-PCR) analyses using primer-sets specific for *Paramyxovirinae*[9], hPIV 1-4, sendai virus, simian virus type 5, New-Castle disease virus, hRSV, morbilli, mumps, Nipah, Hendra, Tupaia and Mapuera viruses. RT-PCR assays were carried out at low stringency in order to detect potentially related viruses and RNA isolated from homologous virus stocks were used as controls. Whereas the available controls reacted positive with the respective virus-specific primers, the newly identified

virus isolates did not react with any primer set, indicating the virus was not closely related to the viruses tested.

We used two of the virus-infected tMK cell culture supernatants to inoculate guinea pigs and ferrets intranasaly. Sera were collected from these animals at day zero, two weeks and three weeks post inoculation. The animals displayed no clinical symptoms but all seroconverted as measured in virus neutralisation (VN) assays and indirect IFA against the homologous viruses. The sera did not react in indirect IFA with any of the known paramyxoviruses described above and with PVM. Next, we screened the so far unidentified virus isolates using the guinea pig and ferret pre- and post-infection sera, of which 28 were clearly positive by indirect IFA with the post-infection sera suggesting they were serological closely related or identical.

RAP PCR

To obtain sequence information on the unknown virus isolates, we used a random PCR amplification strategy known as RAP-PCR[10]. To this end, tMK cells were infected with one of the virus isolates (isolate 00-1) as well as with hPIV-1 which served as a control. After both cultures displayed similar levels of CPE, virus in the culture supernatants was purified on continuous 20-60% sucrose gradients. The gradient fractions were inspected for virus-like particles by EM, and RNA was isolated from the fraction containing approximately 50% sucrose, in which nucleocapsids were observed. Equivalent amounts of RNA isolated from both virus fractions were used for RAP-PCR, after which samples were run side by side on a 3% NuSieve agarose gel. Twenty differentially displayed bands specific for the unidentified virus were subsequently purified from the gel, cloned in plasmid pCR2.1 (Invitrogen) and sequenced with vector-specific primers. When we used these sequences to search for homologies against sequences in the Genbank database using the BLAST software (www.ncbi.nlm.nih.gov/BLAST/) 10 out of 20 fragments displayed resemblance to APV/TRTV sequences. These 10 fragments were located in the genes coding for the nucleoprotein (N; fragment 1 and 2), the matrix protein (M; fragment 3), the fusion protein (F; fragment 4, 5, 6, 7,) and the polymerase protein (L; fragment 8,9,10) (Fig.2). We next designed PCR primers to complete the sequence information for the 3' end of the viral genome based on our RAP PCR fragments as well as published leader and trailer sequences for the *Pneumovirinae* [6]. Three fragments were amplified,

of which fragment A spanned the extreme 3' end of the putative N open reading frame (ORF), fragment B spanned the putative phosphoprotein (P) ORF and fragment C closed the gap between the putative M and F ORFs (Fig. 2). Sequence analyses of these three fragments revealed the absence of NS1 and NS2 ORFs at the extreme 3' end of the viral genome and positioning of the putative F ORF immediately adjacent to the putative M ORF. This genomic organisation resembles that of the metapneumovirus APV, which is also consistent with the sequence homology. Overall the translated sequences for the N, P, M and F ORFs showed an average of 30% homology with members of the genus *Pneumovirus* and 66% with members of the genus *Metapneumovirus*. The amino acid homologies found for N showed 20% homology with hRSV and 52% with APV-C, its closest relative genetically. The amino acid sequence for P showed 25% homology with hRSV and 67% with APV-C, M showed 37% with hRSV and 87% with APV-C, F showed 32% homology with hRSV and 80% with APV-C, and the fragments obtained in L showed an average of 33% with hRSV and 55% with APV-C (table 1).

Phylogeny

Although BLAST searches using nucleotide sequences obtained from the unidentified virus isolate revealed homologies primarily with members of the Pneumovirinae, homologies based on protein sequences revealed some resemblance with other paramyxoviruses as well (data not shown). As an indication for the relation between the newly identified virus isolate and members of the *Pneumovirinae*, phylogenetic trees were constructed based on the N, P, M and F ORFs of these viruses. In all four phylogenetic trees, the newly identified virus isolate was most closely related to APV (Fig.4). From the four serotypes of APV that have been described[11], APV serotype C, the metapneumovirus found primarily in birds in the USA, showed the closest resemblance to the newly identified virus. It should be noted however, that only partial sequence information for APV serotype D is available.

To determine the relationship of our various newly identified virus isolates, we constructed phylogenetic trees based on sequence information obtained from eight to nine isolates (8 for F, 9 for N, M and L). To this end, we used RT-PCR with primers designed to amplify short fragments in the N, M, F and L ORFs, that were subsequently sequenced directly. The nine virus isolates

that were previously found to be related in serological terms (see above) were also found to be closely related genetically. In fact, all nine isolates were more closely related to one another than to APV. Although the sequence information used for these phylogenetic trees was limited, it appears that the nine isolates can be divided in two groups, with isolate 94-1, 99-1 and 99-2 clustering in one group and the other six isolates (94-2; 93-1; 93-2; 93-3; 93-4; 00-1) in the other (Fig.5).

Seroprevalence

To study the seroprevalence of this virus in the human population, we tested sera from humans in different age categories by indirect IFA using tMK cells infected with one of the unidentified virus isolates. This analysis revealed that 25% of the children between six and twelve months had antibodies to the virus, and by the age of five nearly 100% of the children were seropositive. In total 56 serum samples tested by indirect IFA were tested by VN assay. For 51 (91%) of the samples the results of the VN assay (titre >8) coincided with the results obtained with indirect IFA (titre>32). Four samples that were found positive in IFA, were negative by VN test (titre <8) whereas one serum reacted negative in IFA (titre<32) and positive in the VN test (titre 16) (table 2). IFA conducted with 72 sera taken from humans in 1958 (ages ranging from 8-99 years)[12,27] revealed a 100% seroprevalence, indicating the virus has been circulating in the human population for more than 40 years. In addition a number of these sera were used in VN assays to confirm the IFA data (table 2).

Genetic analyses of the putative N, M, P and F genes revealed that MPV has higher sequence homology to the recently proposed genus *Metapneumovirinae* (average of 63 %) as compared to the genus *Pneumovirinae* (average of 30 %) and thus demonstrates a genomic organisation similar to and resembling that of APV/TRTV. In contrast to the genomic organisation of the RSVs ('3-NS1-NS2-N-P-M-SH-G-F-M2-L-5'), metapneumoviruses lack NS1 and NS2 genes and have a different positioning of the genes between M and L ('3-N-P-M-F-M2-SH-G-L-5'). The lack of ORFs between the M and F genes in our virus isolates and the lack of NS1 and NS2 adjacent to to N, and the high amino acid sequence homology found with APV are reasons to propose the classification of MPV as first member of the *Metapneumovirus* genus of mammalian, in particular of human origin.

Phylogenetic analyses revealed that the nine MPV isolates from which sequence information was obtained are closely related. Although sequence information was limited, they were in fact more closely related to one another than to any of the avian metapneumoviruses. Of the four serotypes of APV that have been described, serotype C was most closely related to MPV based on the N, P, M and F genes. It should be noted however that for serotype D only partial sequences for the F gene were available from Genbank and for serotype B only M, N and F sequences were available. Our MPV isolates formed two clusters in phylogenetic trees. For both hRSV and APV different genetic and serological subtypes have been described. Whether the two genetic clusters of MPV isolates represent serogical subgroups that are also functionally different remains unknown at present.

Our serological surveys showed that MPV is a common human pathogen. The repeated isolation of this virus from clinical samples from children with severe RTI indicates that the clinical and economical impact of MPV may be high. New diagnostic assays based on virus detection and serology will allow a more detailed analysis of the incidence and clinical and economical impact of this viral pathogen.

The slight differences between the IFA and VN results (5 samples) maybe due to the fact that in the IFA only IgG serum antibodies were detected whereas the VN assay detects both classes and sub-classes of antibodies or differences may be due to the differences in sensitivity between both assays. For IFA a cut off value of 16 is used, whereas for VN a cut off value of 8 is used.

On the other hand, differences between IFA versus VN assay may also indicate possible differences between different serotypes of this newly identified virus. Since MPV seems most closely related to APV, we speculate that the human virus may have originated from birds. Analysis of serum samples taken from humans in 1958 revealed that MPV has been widespread in the human population for more then 40 years indicating that a tentative zoonosis event must have taken place long before 1958.

Materials and Methods

Specimen collection

Over the past decades our laboratory has collected nasopharyngeal aspirates from children suffering from RTI, which are routinely tested for the presence of viruses. All nasopharyngeal aspirates were tested by direct immmunofluorescence assays (DIF) using fluorescence labelled antibodies against influenza virus types A, and B, hRSV and human parainfluenza virus (hPIV) types 1 to 3. The nasopharyngeal aspirates were also processed for virus isolation using rapid shell vial techniques[14] on various celllines including VERO cells, tertiary cynomolgous monkey kidney (tMK) cells, human endothelial lung (HEL) cells and marbin dock kidney (MDCK) cells. Samples showing cytophatic effects (CPE) after two to three passages, and which were negative in DIF, were tested by indirect immunofluorescence assays (IFA) using virus specific antibodies against influenza virus types A, B and C, hRSV types A and B, measles virus, mumps virus, human parainfluenza virus (hPIV) types 1 to 4, sendai virus, simian virus type 5, and New-Castle disease virus. Although for many cases the aetiological agent could be identified, some specimens were negative for all these viruses tested.

Direct Immunofluorescence Assay (DIF)

Nasopharyngeal aspirate samples from patients suffering from RTI were used for DIF and virus isolation as described[14,15]. Samples were stored at -70 °C. In brief, nasopharyngeal aspirates were diluted with 5 ml Dulbecco MEM (BioWhittaker, Walkersville, MD) and thoroughly mixed on a vortex mixer for one minute. The suspension was thus centrifuged for ten minutes at 840 x *g*. The sediment was spread on a multispot slide (Nutacon, Leimuiden, The Netherlands), the supernatant was used for virus isolation. After drying, the cells were fixed in aceton for 1 minute at room temperature. After washing the slides were incubated for 15 minutes at 37 °C with commercial available FITC-labelled virus specific anti-sera such as influenza A and B, hRSV and hPIV 1 to 3 (Dako, Glostrup, Denmark). After three washings in PBS and one in tap water, the slides were included in a glycerol/PBS solution (Citifluor, UKC,

Canterbury, UK) and covered. The slides were analysed using a Axioscop fluorescence microscope (Carl Zeiss B.V, Weesp, the Netherlands.

Virus isolation

For virus isolation tMK cells (RIVM, Bilthoven, The Netherlands) were cultured in 24 well plates containing glass slides (Costar, Cambridge, UK), with the medium described below supplemented with 10% fetal bovine serum (BioWhittaker, Vervier, Belgium). Before inoculation the plates were washed with PBS and supplied with Eagle's MEM with Hanks' salt (ICN, Costa mesa, CA) of which half a litre was supplemented with 0.26 gram $HaHCO_3$, 0.025 M Hepes (Biowhittaker), 2 mM L-glutamine (Biowhittaker), 100 units penicilline, 100 µg streptomycine (Biowhittaker), 0.5 gram lactalbumine (Sigma-Aldrich, Zwijndrecht, The Netherlands), 1.0 gram D-glucose (Merck, Amsterdam, The Netherlands), 5.0 gram peptone (Oxoid, Haarlem, The Netherlands) and 0.02% trypsine (Life Technologies, Bethesda, MD). The plates were inoculated with supernatant of the nasopharyngeal aspirate samples, 0,2 ml per well in triplicate, followed by centrifuging at 840x *g* for one hour. After inoculation the plates were incubated at 37 °C for a maximum of 14 days changing the medium once a week and cultures were checked daily for CPE. After 14 days cells were scraped from the second passage and incubated 14 days. This step was repeated for the third passage. The glass slides were used to demonstrate the presence of the virus by indirect IFA as described below.

Animal immunisation

Ferret and guinea pig specific antisera for the newly discovered virus were generated by experimental intranasal infection of two specific pathogen free ferrets and two guinea pigs, housed in separate pressurised glove boxes. Two to three weeks later all the animals were bled by cardiac puncture, and their sera were used as reference sera. The sera were tested for all previous described viruses with indirect IFA as described below.

Antigen detection by indirect IFA

We performed indirect IFA on slides containing infected tMK cells. After washing with PBS the slides were incubated for 30 minutes at 37 °C with virus specific anti-sera. We used monoclonal antibodies in DIF against influenza A, B

and C, hPIV type 1 to 3 and hRSV as described above. For hPIV type 4, mumps virus, measles virus, sendai virus, simian virus type 5, New-Castle Disease virus polyclonal antibodies (RIVM) and ferret and guinea pig reference sera were used. After three washings with PBS and one wash with tap water, the slides were stained with a secondary antibodies directed against the sera used in the first incubation. Secondary antibodies for the polyclonal anti sera were goat-anti-ferret (KPL, Guilford, UK, 40 fold diluted), mouse-anti-rabbit (Dako, Glostrup, Denmark, 20 fold diluted), rabbit-anti-chicken (KPL, 20 fold dilution) and mouse-anti-guinea pig (Dako, 20 fold diluted). Slides were processed as described for DIF.

Detection of antibodies in humans by indirect IFA

For the detection of virus specific antibodies, infected tMK cells were fixed with cold acetone on coverslips, washed with PBS and stained with serum samples at a 1 to 16 dilution. Subsequently, samples were stained with FITC-labelled rabbit anti human antibodies 80 times diluted in PBS (Dako). Slides were processed as described above.

Virus culture of hMPV

Sub-confluent mono-layers of tMK cells in media as described above were inoculated with supernatants of samples that displayed CPE after two or three passages in the 24 well plates. Cultures were checked for CPE daily and the media was changed once a week. Since CPE differed for each isolate, all cultures were tested at day 12 to 14 with indirect IFA using ferret antibodies against the new virus isolate. Positive cultures were freeze-thawed three times, after which the supernatants were clarified by low-speed centrifugation, aliquoted and stored frozen at -70 °C. The 50% tissue culture infectious doses (TCID50) of virus in the culture supernatants were determined as described[16].

Virus neutralisation assay

VN assays were performed with serial two-fold dilutions of human and animal sera starting at an eight-fold dilution. Diluted sera were incubated for one hour with 100 TCID50 of virus before inoculation of tMK cells grown in 96 well plates, after which the plates were centrifuged at 840 x *g*. The media was changed after three and six days and IFA was conducted with ferret antibodies against hMPV

8 days after inoculation. The VN titre was defined as the lowest dilution of the serum sample resulting in negative IFA and inhibition of CPE in cell cultures.

Virus characterisation

Haemagglutination assays and chloroform sensitivity tests were performed as described[8,14]. For EM analyses, virus was concentrated from infected cell culture supernatants in a micro-centrifuge at 4 °C at 17000 x g, after which the pellet was resuspended in PBS and inspected by negative contrast EM. For RAP-PCR, virus was concentrated from infected tMK cell supernatants by ultra-centrifugation on a 60% sucrose cussion (2 hours at 150000 x g, 4 °C). The 60% sucrose interphase was subsequently diluted with PBS and layered on top of a 20-60% continuous sucrose gradient which was centrifuged for 16 hours at 275000 x g at 4 °C. Sucrose gradient fractions were inspected for the presence of virus-like particles by EM and poly-acrylamide gel electrophoresis followed by silver staining. The approximately 50% sucrose fractions that appeared to contain nucleocapsids were used for RNA isolation and RAP-PCR.

RNA isolation

RNA was isolated from the supernatant of infected cell cultures or sucrose gradient fractions using a High Pure RNA Isolation kit according to instructions from the manufacturer (Roche Diagnostics, Almere, The Netherlands).

RT-PCR

Virus-specific oligonucleotide sequences for RT-PCR assays on known paramyxoviruses are described in addenda 1. A one-step RT-PCR was performed in 50 µl reactions containing 50 mM Tris.HCl pH 8.5, 50 mM NaCl, 4 mM $MgCl_2$, 2 mM dithiotreitol, 200 µM each dNTP, 10 units recombinant RNAsin (Promega, Leiden, the Netherlands), 10 units AMV RT (Promega, Leiden, The Netherlands), 5 units Amplitaq Gold DNA polymerase (PE Biosystems, Nieuwerkerk aan de Ijssel, The Netherlands) and 5 µl RNA. Cycling conditions were 45 min. at 42 °C and 7 min. at 95 °C once, 1 min at 95 °C, 2 min. at 42 °C and 3 min. at 72 °C repeated 40 times and 10 min. at 72 °C once.

RAP-PCR

RAP-PCR was performed essentially as described[10]. The oligonucleotide sequences are described in addenda 2. For the RT reaction, 2 µl RNA was used in a 10 µl reaction containing 10 ng/µl oligonucleotide, 10 mM dithiotreitol, 500 µm each dNTP, 25 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM $MgCl_2$. The reaction mixture was incubated for 5 min. at 70 °C and 5 min. at 37 °C, after which 200 units Superscript RT enzyme (LifeTechnologies) were added. The incubation at 37 °C was continued for 55 min. and the reaction terminated by a 5 min. incubation at 72 °C. The RT mixture was diluted to give a 50 µl PCR reaction containing 8 ng/µl oligonucleotide, 300 µm each dNTP, 15 mM Tris-HCL pH 8.3, 65 mM KCl, 3.0 mM $MgCL_2$ and 5 units Taq DNA polymerase (PE Biosystems). Cycling conditions were 5 min. at 94 °C, 5 min. at 40 °C and 1 min. at 72 °C once, followed by 1 min. at 94 °C, 2 min. at 56 °C and 1 min. at 72 °C repeated 40 times and 5 min. at 72°C once. After RAP-PCR, 15 µl the RT-PCR products were run side by side on a 3% NuSieve agarose gel (FMC BioProducts, Heerhugowaard, The Netherlands). Differentially displayed fragments specific for hMPV were purified from the gel with Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and cloned in pCR2.1 vector (Invitrogen, Groningen, The Netherlands) according to instructions from the manufacterer.

Sequence analysis

RAP-PCR products cloned in vector pCR2.1 (Invitrogen) were sequenced with M13-specific oligonucleotides. DNA fragments obtained by RT-PCR were purified from agarose gels using Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands), and sequenced directly with the same oligonucleotides used for PCR. Sequence analyses were performed using a Dyenamic ET terminator sequencing kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI 373 automatic DNA sequencer (PE Biosystem). All techniques were performed according to the instructions of the manufacturer.

Generating genomic fragments of MPV by RT-PCR

To generate PCR fragments spanning gaps A, B and C between the RAP-PCR fragments (Fig.2) we used RT-PCR assays as described before on RNA isolated from virus isolate 00-1. The following primers were used:
For fragment A: TR1 designed in the leader: (5'-AAAGAATTCACGAGAAAAAAACGC-3') and N1 designed at the 3'end of the RAP-PCR fragments obtained in N (5'-CTGTGGTCTCTAGTCCCACTTC-3')
For fragment B: N2 designed at the 5'end of the RAP-PCR fragments obtained in N: (5'-CATGCAAGCTTATGGGGC-3') and M1 designed at the 3'end of the RAP-PCR fragments obtained in M: (5'-CAGAGTGGTTATTGTCAGGGT-3').
For fragment C: M2 designed at the 5'end of the RAP-PCR fragment obtained in M: (5'-GTAGAACTAGGAGCATATG-3') and F1 designed at the 3'end of the RAP-PCR fragments obtained in F: (5'-TCCCCAATGTAGATACTGCTTC-3').
Fragments were purified from the gel, cloned and sequenced as described before.

RT-PCR for diagnosing MPV.

For the amplification and sequencing of parts of the N, M, F and L ORFs of nine of the hMPV isolates, we used primers N3 (5'-GCACTCAAGAGATACCCTAG -3') and N4 (5'-AGACTTTCTGCTTTGCTGCCTG-3'), amplifying a 151 nucleotide fragments, M3 (5'-CCCTGACAATAACCACTCTG-3') and M4 (5'-GCCAACTGATTTGGCTGAGCTC-3') amplifying a 252 nucleotide fragment, F7 (5'-TGCACTATCTCCTCTTGGGGCTTTG-3') and F8 (5'-TCAAAGCTGCTTGACACTGGCC-3') amplifying a 221 nucleotide fragment and L6 (5'-CATGCCCACTATAAAAGGTCAG-3') and L7 (5'-CACCCCAGTCTTTCTTGAAA-3') amplifying a 173 nucleotide fragment respectively. RT-PCR, gel purification and direct sequencing were performed as described above. Furthermore, probes used were:

Probe used in M: 5'-TGC TTG TAC TTC CCA AAG-3'
Probe used in N: 5'-TAT TTG AAC AAA AAG TGT-3'
Probe used in L: 5'-TGGTGTGGGATATTAACAG-3'

Phylogenetic analyses

For all phylogenetic trees, DNA sequences were alligned using the ClustalW software package and maximum likelihood trees were generated using the DNA-ML software package of the Phylip 3.5 program using 100 bootstraps and 3 jumbles[15]. Previously published sequences that were used for the generation of phylogenetic trees are available from Genbank under accessions numbers : For all ORFs: hRSV: NC001781; bRSV: NC001989; For the F ORF: PVM, D11128; APV-A, D00850; APV-B, Y14292; APV-C, AF187152; For the N ORF: PVM, D10331; APV-A, U39295; APV-B, U39296; APV-C, AF176590; For the M ORF: PMV,U66893; APV-A, X58639; APV-B, U37586; APV-C, AF262571; For the P ORF: PVM, 09649; APV-A, U22110, APV-C, AF176591. Phylogenetic analyses for the nine different virus isolates of hMPV were performed with APV strain C as outgroup.

Abbreviations used in figures: hRSV: human RSV; bRSV: bovine RSV; PVM: pneumonia virus of mice; APV-A, B,and C: avian pneumovirus typ A, B and C.

Examples of methods to identify MPV

Specimen collection

In order to find virus isolates nasopharyngeal aspirates, throat and nasal swabs, broncheo alveolar lavages preferably from mammals such as humans, carnivors (dogs, cats, mustellits, seals etc.), horses, ruminants (cattle, sheep, goats etc.), pigs, rabbits, birds (poultry, ostridges, etc) should be examined. From birds cloaca swabs and droppings can be examined as well. Sera should be collected for immunological assays, such as ELISA and virus neutralisation assays.
Collected virus specimens were diluted with 5 ml Dulbecco MEM medium (BioWhittaker, Walkersville, MD) and thoroughly mixed on a vortex mixer for one minute. The suspension was thus centrifuged for ten minutes at 840 x g. The sediment was spread on a multispot slide (Nutacon, Leimuiden, The Netherlands) for immunofluorescence techniques, and the supernatant was used for virus isolation.

Virus isolation

For virus isolation tMK cells (RIVM, Bilthoven, The Netherlands) were cultured in 24 well plates containing glass slides (Costar, Cambridge, UK), with the medium described below supplemented with 10% fetal bovine serum (BioWhittaker, Vervier, Belgium). Before inoculation the plates were washed with PBS and supplied with Eagle's MEM with Hanks' salt (ICN, Costa mesa, CA) supplemented with 0.52/liter gram $NaHCO_3$, 0.025 M Hepes (Biowhittaker), 2 mM L-glutamine (Biowhittaker), 200 units/liter penicilline, 200 µg/liter streptomycine (Biowhittaker), 1gram/liter lactalbumine (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2.0 gram/liter D-glucose (Merck, Amsterdam, The Netherlands), 10 gram/liter peptone (Oxoid, Haarlem, The Netherlands) and 0.02% trypsine (Life Technologies, Bethesda, MD). The plates were inoculated with supernatant of the nasopharyngeal aspirate samples, 0,2 ml per well in triplicate, followed by centrifuging at 840x *g* for one hour. After inoculation the plates were incubated at 37 °C for a maximum of 14 days changing the medium once a week and cultures were checked daily for CPE. After 14 days, cells were scraped from the second passage and incubated for another 14 days. This step was repeated for the third passage. The glass slides were used to demonstrate the presence of the virus by indirect IFA as described below.
CPE was generally observed after the third passage, at day 8 to 14 depending on the isolate. The CPE was virtually indistinghuisable from that caused by hRSV or hPIV in tMK or other cell cultures. However, hRSV induces CPE starting around day 4. CPE was characterised by syncytia formation, after which the cells showed rapid internal disruption, followed by detachment of cells from the monolayer. For some isolates CPE was difficult to observe, and IFA was used to confirm the presence of the virus in these cultures.

Virus culture of hMPV

Sub-confluent monolayers of tMK cells in media as described above were inoculated with supernatants of samples that displayed CPE after two or three passages in the 24 well plates. Cultures were checked for CPE daily and the media was changed once a week. Since CPE differed for each isolate, all cultures were tested at day 12 to 14 with indirect IFA using ferret antibodies against the new virus isolate. Positive cultures were freeze-thawed three times, after which

the supernatants were clarified by low-speed centrifugation, aliquoted and stored frozen at -70 °C. The 50% tissue culture infectious doses (TCID50) of virus in the culture supernatants were determined following established techniques used in the field[16].

Virus characterisation

Haemagglutination assays and chloroform sensitivity tests were performed following well established and described techniques used in the field[14]. For EM analyses, virus was concentrated from infected cell culture supernatants in a micro-centrifuge at 4 °C at 17000 x g, after which the pellet was resuspended in PBS and inspected by negative contrast EM.

Antigen detection by indirect IFA

Collected specimens were processed as described and sediment of the samples was spread on a multispot slide. After drying, the cells were fixed in aceton for 1 minute at room temperature.

Alternatively, virus was cultured on tMK cells in 24 well slides containing glass slides. These glass slides were washed with PBS and fixed in aceton for 1 minute at room temperature.

After washing with PBS the slides were incubated for 30 minutes at 37 °C with polyclonal antibodies at a dilution of 1:50 to 1:100 in PBS. We used immunised ferrets and guinea pigs to obtain polyclonal antibodies, but these antibodies can be raised in various animals, and the working dilution of the polyclonal antibody can vary for each immunisation. After three washes with PBS and one wash with tap water, the slides were incubated at 37°C for 30 minutes with FITC labeled goat-anti-ferret antibodies (KPL, Guilford, UK, 40 fold diluted). After three washes in PBS and one in tap water, the slides were included in a glycerol/PBS solution (Citifluor, UKC, Canterbury, UK) and covered. The slides were analysed using an Axioscop fluorescence microscope (Carl Zeiss B.V., Weesp, the Netherlands).

Detection of antibodies in humans, mammals, ruminants or other animals by indirect IFA

For the detection of virus specific antibodies, infected tMK cells with MPV were fixed with acetone on coverslips (as described above), washed with PBS and incubated 30 minutes at 37°C with serum samples at a 1 to 16 dilution. After two washes with PBS and one with tap water, the slides were incubated 30 minutes at 37°C with FITC-labelled secondary antibodies to the species used (Dako). Slides were processed as described above.

Antibodies can be labelled directly with a fluorescent dye, which will result in a direct immuno fluorescence assay. FITC can be replaced with any fluorescent dye.

Animal immunisation

Ferret and guinea pig specific antisera for the newly discovered virus were generated by experimental intranasal infection of two specific pathogen free ferrets and two guinea pigs, housed in separate pressurised glove boxes. Two to three weeks later the animals were bled by cardiac puncture, and their sera were used as reference sera. The sera were tested for all previous described viruses with indirect IFA as described below. Other animal species are also suitable for the generation of specific antibody preparations and other antigen preparations may be used.

Virus neutralisation assay (VN assay)

VN assays were performed with serial two-fold dilutions of human and animal sera starting at an eight-fold dilution. Diluted sera were incubated for one hour with 100 TCID50 of virus before inoculation of tMK cells grown in 96 well plates, after which the plates were centrifuged at 840 x *g*. The same culture media as described above was used. The media was changed after three and six days, and after 8 days IFA was performed (see above). The VN titre was defined as the lowest dilution of the serum sample resulting in negative IFA and inhibition of CPE in cell cultures.

RNA isolation

RNA was isolated from the supernatant of infected cell cultures or sucrose gradient fractions using a High Pure RNA Isolation kit according to instructions from the manufacturer (Roche Diagnostics, Almere, The Netherlands). RNA can also be isolated following other procedures known in the field (*Current Protocols in Molecular Biology*).

RT-PCR

A one-step RT-PCR was performed in 50 µl reactions containing 50 mM Tris.HCl pH 8.5, 50 mM NaCl, 4 mM $MgCl_2$, 2 mM dithiotreitol, 200 µM each dNTP, 10 units recombinant RNAsin (Promega, Leiden, the Netherlands), 10 units AMV RT (Promega, Leiden, The Netherlands), 5 units Amplitaq Gold DNA polymerase (PE Biosystems, Nieuwerkerk aan de Ijssel, The Netherlands) and 5 µl RNA. Cycling conditions were 45 min. at 42 °C and 7 min. at 95 °C once, 1 min at 95 °C, 2 min. at 42 °C and 3 min. at 72 °C repeated 40 times and 10 min. at 72 °C once.

Primers used for diagnostic PCR:

In the nucleoprotein: N3 (5'-GCACTCAAGAGATACCCTAG -3') and N4 (5'-AGACTTTCTGCTTTGCTGCCTG-3'), amplifying a 151 nucleotide fragment.

In the matrixprotein: M3 (5'-CCCTGACAATAACCACTCTG-3') and M4 (5'-GCCAACTGATTTGGCTGAGCTC-3') amplifying a 252 nucleotide fragment

In the polymerase protein: L6 (5'-CATGCCCACTATAAAAGGTCAG-3') and L7 (5'-CACCCCAGTCTTTCTTGAAA-3') amplifying a 173 nucleotide fragment.

Other primers can be designed based on MPV sequences, and different buffers and assay conditions may be used for specific purposes.

Sequence analysis

Sequence analyses were performed using a Dyenamic ET terminator sequencing kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI 373 automatic DNA sequencer (PE Biosystem). All techniques were performed according to the instructions of the manufacturer. PCR fragments were sequenced directly with the same oligonucleotides used for PCR, or the fragments were purified from the gel with Qiaquick Gel Extraction kit (Qiagen,

Leusden, The Netherlands) and cloned in pCR2.1 vector (Invitrogen, Groningen, The Netherlands) according to instructions from the manufacturer and subsequently sequenced with M13-specific oligonucleotides.

Oligonucleotides used for analysing the 3'end of the genome (absence of NS1/NS2).

Primer TR1 (5'-AAAGAATTCACGAGAAAAAAACGC-3') was designed based on published sequences of the trailer and leader for hRSV and APV, published by Randhawa (1997) and primer N1 (5'-CTGTGGTCTCTAGTCCCACTTC-3') was designed based on obtained sequences in the N protein. The RT-PCR assay and sequencing was performed as described above.

The RT-PCR gave a product of approximately 500 base pairs which is to small to contain information for two ORFS, and translation of these sequences did not reveal an ORF.

Detection of antibodies in humans, mammals, ruminants or other animals by ELISA

In *Paramyxoviridae,* the N protein is the most abundant protein, and the immune response to this protein occurs early in infection. For these reasons, a recombinant source of the N proteins is preferably used for developing an ELISA assay for detection of antibodies to MPV. Antigens suitable for antibody detection include any MPV protein that combines with any MPV-specific antibody of a patient exposed to or infected with MPV virus. Preferred antigens of the invention include those that predominantly engender the immune response in patients exposed to MPV, which therefore, typically are recognised most readily by antibodies of a patient. Particularly preferred antigens include the N, F and G proteins of MPV.

Antigens used for immunological techniques can be native antigens or can be modified versions thereof. Well known techniques of molecular biology can be used to alter the amino acid sequence of a MPV antigen to produce modified versions of the antigen that may be used in immunologic techniques.

Methods for cloning genes, for manipulating the genes to and from expression vectors, and for expressing the protein encoded by the gene in a heterologous host are well-known, and these techniques can be used to provide the expression

vectors, host cells, and the for expressing cloned genes encoding antigens in a host to produce recombinant antigens for use in diagnostic assays. See for instance: *Molecular cloning, A laboratory manual* and *Current Protocols in Molecular Biology*.

A variety of expression systems may be used to produce MPV antigens. For instance, a variety of expression vectors suitable to produce proteins in *E.Coli*, *B.subtilis*, yeast, insect cells and mammalian cells have been described, any of which might be used to produce a MPV antigen suitable to detect anti-MPV antibodies in exposed patients.

The baculovirus expression system has the advantage of providing necessary processing of proteins, and is therefor preferred. The system utilizes the polyhedrin promoter to direct expression of MPV antigens. (Matsuura et al. 1987, J.Gen.Virol. 68: 1233-1250).

Antigens produced by recombinant baculo-viruses can be used in a variety of immunological assays to detect anti-MPV antibodies in a patient. It is well established, that recombinant antigens can be used in place of natural virus in practically any immunological assay for detection of virus specific antibodies. The assays include direct and indirect assays, sandwich assays, solid phase assays such as those using plates or beads among others, and liquid phase assays. Assays suitable include those that use primary and secondary antibodies, and those that use antibody binding reagents such as protein A. Moreover, a variety of detection methods can be used in the invention, including colorimetric, fluorescent, phosphorescent, chemiluminescent, luminescent and radioactive methods.

Example 1 of indirect anti-PMV IgG EIA using recombinant N protein

An indirect IgG EIA using a recombinant N protein (produced with recombinant baculo-virus in insect (Sf9) cells) as antigen can be performed. For antigen preparation, Sf9 cells are infected with the recombinant baculovirus and harvested 3-7 days post infection. The cell suspension is washed twice in PBS, pH 7.2, adjusted to a cell density of 5.0X 10^6 cells/ml, and freeze-thawed three times. Large cellular debris is pelleted by low speed centrifugation (500 x g for 15 min.) and the supernatant is collected and stored at -70°C until use. Uninfected cells are processed similarly for negative control antigen.

100 µl of a freeze-thaw lysate is used to coat microtiter plates, at dilutions ranging from 1:50 to 1:1000. An uninfected cell lysate is run in duplicate wells and serves as a negative control. After incubation overnight, plates are washed twice with PBS/0.05%Tween.Test sera are diluted 1:50 to 1:200 in ELISA buffer (PBS, supplemented to 2% with normal goat sera, and with 0.5% bovine serum albumine and 0.1% milk), followed by incubation wells for 1 hour at 37°C.

Plates are washed two times with PBS/0.05%Tween. Horseradish peroxidase labelled goat anti-human (or against other species) IgG, diluted 1:3000 to 1:5000 in ELISA buffer, added to wells, and incubated for 1 hour at 37°. The plates are then washed two times with PBS/0.05%Tween and once with tap water, incubated for 15 minutes at room temperature with the enzyme substrate TMB, 3,3',5,5' tetramethylbenzidine, such as that obtained from Sigma, and the reaction is stopped with 100 µl of 2 M phosphoric acid. Colorimetric readings are measured at 450 nm using an automated microtiter plate reader.

Example 2: Capture anti-MPV IgM EIA using a recombinant nucleoprotein

A capture IgM EIA using the recombinant nucleoprotein or any other recombinant protein as antigen can be performed by modification of assays as previously described by Erdman et al (1990) J.Clin.Microb. 29: 1466-1471. Affinity purified anti-human IgM capture antibody (or against other species), such as that obtained from Dako, is added to wells of a microtiter plate in a concentration of 250 ng per well in 0.1 M carbonate buffer pH 9.6. After overnight incubation at room temperature, the plates are washed two times with PBS/0.05% Tween. 100 µl of test serum diluted 1:200 to 1:1000 in ELISA buffer is added to triplicate wells and incubated for 1 hour at 37°C. The plates are then washed two times with in PBS/0.05%Tween.

The freeze-thawed (infected with recombinant virus) Sf21 cell lysate is diluted 1:100 to 1: 500 in ELISA buffer is added to the wells and incubated for 2 hours at 37°C. Uninfected cell lysate serves as a negative control and is run in duplicate wells.

The plates are then washed three times in PBS/0.05% Tween and incubated for 1 hour at 37°C with 100 µl of a polyclonal antibody against MPV in a optimal

dilution in ELISA buffer. After 2 washes with PBS/0.05% Tween , the plates are incubated with horseradish peroxide labeled secondary antibody (such as rabbit anti ferret), and the plates are incubated 20 minutes at 37°C.
The plates are then washed five times in PBS/0/05% Tween, incubated for 15 minutes at room temperature with the enzyme substrate TMB, 3,3',5,5' tetramethylbenzidine, as, for instance obtained from "Sigma", and the reaction is stopped with 100 µl of 2M phosphoric acid. Colormetric readings are measured at 450 nm using automated microtiter plate reader.

The sensitivities of the capture IgM EIAs using the recombinant nucleoprotein (or other recombinant protein) and whole MPV virus are compared using acute- and convalescent-phase serum pairs form persons with clinical MPV virus infection. The specificity of the recombinant nucleoprotein capture EIA is determined by testing serum specimens from healthy persons and persons with other paramyxovirus infections.

Potential for EIAs for using recombinant MPV fusion and glycoprotein proteins produced by the baculovirus expression.

The glycoproteins G and F are the two transmembraneous envelope glycoproteins of the MPV virion and represent the major neutralisation and protective antigens.
The expression of these glycoproteins in a vector virus system sych as a baculovirus system provides a source of recombinant antigens for use in assays for detection of MPV specific antibodies. Moreover, their use in combination with the nucleoprotein, for instance, further enhances the sensitivity of enzyme immunoassays in the detection of antibodies against MPV.

A variety of other immunological assays (*Current Protocols in Immunology*) may be used as alternative methods to those described here.
In order to find virus isolates nasopharyngeal aspirates, throat and nasal swabs, broncheo alveolar lavages and throat swabs preferable from but not limited to humans, carnivors (dogs, cats, mustellits, seals etc.), horses, ruminants (cattle, sheep, goats etc.), pigs, rabbits, birds (poultry, ostridges, etc) can be examined. From birds, cloaca and intestinal swabs and droppings can be examined as well.

For all samples, serology (antibody and antigen detection etc.), virus isolation and nucleic acid detection techniques can be performed for the detection of virus. Monoclonal antibodies can be generated by immunising mice (or other animals) with purified MPV or parts thereof (proteins, peptides) and subsequently using established hybridoma technology (*Current protocols in Immunology*). Alternatively, phage display technology can be used for this purpose (*Current protocols in Immunology*).
Similarly, polyclonal antibodies can be obtained from infected humans or animals, or from immunised humans or animals (*Current protocols in Immunology*).

The detection of the presence or absence of NS1 and NS2 proteins can be performed using western-blotting, IFA, immuno precipitation techniques using a variety of antibody preparations. The detection of the presence or absence of NS1 and NS2 genes or homologues thereof in virus isolates can be performed using PCR with primer sets designed on the basis of known NS1 and/or NS2 genes as well as with a variety of nucleic acid hybridisation techniques.

To determine whether NS1 and NS2 genes are present at the 3' end of the viral genome, a PCR can be performed with primers specific for this 3' end of the genome. In our case, we used a primer specific for the 3' untranslated region of the viral genome and a primer in the N ORF. Other primers may be designed for the same purpose. The absence of the NS1/NS2 genes is revealed by the length and/or nucleotide sequence of the PCR product. Primers specific for NS1 and/or NS2 genes may be used in combination with primers specific for other parts of the 3' end of the viral genome (such as the untranslated region or N ORF) to allow a positive identification of the presence of NS1 or NS2 genes. In addition to PCR, a variety of techniques such as molecular cloning, nucleic acid hybridisation may be used for the same purpose.

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Primers used for RT-PCR detection of known paramyxo-viruses. Primers for hPIV-1 to 4, mumps, measles, Tupaia, Mapuera and Hendra are developed in house and based on allignments of available sequences. Primers for New Castle Disease Virus are taken from Seal, J., J. et al; Clin. Microb., 2624-2630, 1995. Primers for Nipah and general paramyxovirus-PCR are taken from: Chua, K.B., et al; Science, 288 26 may 2000

Virus protein		primers	located in
HPIV-1	fwd	5'-TGTTGTCGAGACTATTCCAA-3'	HN
	Rev	5'-TGTTG(T/A)ACCAGTTGCAGTCT-3'	
HPIV-2	Fwd	5'-TGCTGCTTCTATTGAGAAACGCC-3'	N
	Rev	5'-GGTGAC/T TC(T/C)AATAGGGCCA-3'	
HPIV-3	Fwd	5'-CTCGAGGTTGTCAGGATATAG-3'	HN
	Rev	5'-CTTTGGGAGTTGAACACAGTT-3'	
HPIV-4	Fwd	5'-TTC(A/G)GTTTTAGCTGCTTACG-3'	N
	Rev	5'-AGGCAAATCTCTGGATAATGC-3'	
Mumps	Fwd	5'-TCGTAACGTCTCGTGACC-3'	SH
	Rev	5'-GGAGATCTTTCTAGAGTGAG-3'	
NDV	Fwd	5'-CCTTGGTGAiTCTATCCGIAG-3'	F
	Rev	5'-CTGCCACTGCTAGTTGiGATAATCC-3'	
Tupaia	Fwd	5'-GGGCTTCTAAGCGACCCAGATCTTG-3'	N
	Rev	5'-GAATTTCCTTATGGACAAGCTCTGTGC-3'	
Mapuera	Fwd	5'-GGAGCAGGAACTCCAAGACCTGGAG-3'	N
	Rev:	5'-GCTCAACCTCATCACATACTAACCC-3'	
Hendra	Fwd	5'-GAGATGGGCGGGCAAGTGCGGCAACAG-3'	N
	Rev	5'-GCCTTTGCAATCAGGATCCAAATTTGGG-3'	
Nipah	Fwd	5'-CTGCTGCAGTTCAGGAAACATCAG-3'	N
	Rev	5'-ACCGGATGTGCTCACAGAACTG-3'	
HRSV	Fwd	5'-TTTGTTATAGGCATATCATTG-3'	F
	Rev	5'-TTAACCAGCAAAGTGTTA-3'	
Measles	Fwd	5'-TTAGGGCAAGAGATGGTAAGG-3'	N
	Rev	5'-TTATAACAATGATGGAGGG-3'	
General Paramyxoviridae :			
	Fwd	5'-CATTAAAAAGGGCACAGACGC-3'	P
	Rev	5'-TGGACATTCTCCGCAGT-3'	

Primers for RAP-PCR:

ZF1: 5'-CCCACCACCAGAGAGAAA-3'
ZF4: 5'-ACCACCAGAGAGAAACCC-3'
ZF7: 5'-ACCAGAGAGAAACCCACC-3'
ZF10: 5'-AGAGAGAAACCCACCACC-3'
ZF13: 5'-GAGAAACCCACCACCAGA-3'
ZF16: 5'-AAACCCACCACCAGAGAG-3'

CS1: 5'-GGAGGCAAGCGAACGCAA-3'
CS4: 5'-GGCAAGCGAACGCAAGGA-3'
CS7: 5'-AAGCGAACGCAAGGAGGC-3'
CS10:5'-CGAACGCAAGGAGGCAAG-3'
CS13:5'-ACGCAAGGAGGCAAGCGA-3'
CS16:5'-CAAGGAGGCAAGCGAACG-3'

20 fragments successfully purified and sequenced:

10 fragments found with sequence homology in APV

Fragment 1	ZF 7, 335 bp	N gene
Fragment 2	ZF 10, 235 bp	N gene
Fragment 3	ZF 10, 800 bp	M gene
Fragment 4	CS 1, 1250 bp	F gene
Fragment 5	CS 10, 400 bp	F gene
Fragment 6	CS 13, 1450 bp	F gene
Fragment 7	CS 13, 750 bp	F gene
Fragment 8	ZF 4, 780 bp	L gene (protein level)
Fragment 9	ZF 10, 330 bp	L gene (protein level)
Fragment 10	ZF10, 250bp	L gene (protein level)

Primers used for RAP-PCR amplification of nucleic acids from the prototype isolate.

Addendum

Background information on *Pneumovirinae*

The family of *Paramyxoviridae* contains two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae*. The subfamily *Pneumovirinae* consists of two genera: *Pneumovirus* and *Metapneumovirus*. The genus *Pneumovirus* contains the human, bovine, ovine and caprine respiratory syncytial viruses and the pneumonia virus of mice (PVM). The genus *Metapneumovirus* contains the avian pneumoviruses (APV, also referred to as TRTV).

The classification of the genera in the subfamily *Pneumovirinae* is based on classical virus characteristics, gene order and gene constellation. Viruses of the genus *Pneumovirus* are unique in the family of *Paramyxoviridae* in having two nonstructural proteins at the 3'end of the genome (3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'). In contrast, viruses in the genus *Metapneumovirus* lack the NS1 and NS2 genes and the organisation of genes between the M and L coding regions is different: 3'-N-P-M-F-M2-SH-G-L-5'.

All members of the subfamily *Paramyxovirinae* have haemagluttinating activity, but this function is not a defining feature for the subfamily *Pneumovirinae*, being absent in RSV and APV but present in PMV. Neuraminidase activity is present in members of the genera *Paramyxovirus* and *Rubulavirus* (subfamily *Paramyxovirinae*) but is absent in the genus *Morbillivirus* (subfamily *Paramyxovirinae*) and the genera *Pneumovirus* and *Metapneumovirus* (subfamily *Pneumovirinae*).

A second distinguishing feature of the subfamily *Pneumovirinae* is the apparent limited utilization of alternative ORFs within mRNA by RSV. In contrast, several members of the subfamily *Paramyxovirinae*, such as Sendai and Measles viruses, access alternative ORFs within the mRNA encoding the phosphoprotein (P) to direct the synthesis of a novel protein.

The G protein of the *Pneumovirinae* does not have sequence relatedness or structural similarity to the HN or H proteins of *Paramyxovirinae* and is only approximately half the size of their chain length. In addition, the N and P proteins are smaller than their counterparts in the *Paramyxovirinae* and lack unambigous sequence homology. Most nonsegmented negative stranded RNA

viruses have a single matrix (M) protein. Members of the subfamily *Pneumovirinae* are an exception in having two such proteins, M and M2. The M protein is smaller than its *Paramyxovirinae* counterparts and lacks sequence relatedness with *Paramyxovirinae.*

When grown in cell cultures, members of the subfamily *Pneumovirinae* show typical cytopathic effects; they induce characteristic syncytia formation of cells. (Collins, 1996).

The subfamily *Pneumovirinae*, genus *Pneumovirus*

hRSV is the type-species of the genus *Pneumovirus* and is a major and widespread cause of lower respiratory tract illness during infancy and early childhood (Selwyn, 1990). In addition, hRSV is increasingly recognised as an important pathogen in other patient groups, including immune compromised individuals and the elderly. RSV is also an important cause of community-acquired pneumonia among hospitalised adults of all ages (Englund, 1991; Falsey, 2000; Dowell, 1996).

Two major antigenic types for RSV (A and B) have been identified based on differences in their reactivity with monoclonal and polyclonal antibodies and by nucleic acid sequence analyses (Anderson, 1985; Johnson, 1987; Sullender, 2000). In particular the G protein is used in distinguishing the two subtypes. RSV-A and B share only 53% amino acid sequence homology in G, whereas the other proteins show higher homologies between the subtypes (table 1) (Collins, 1996).

Detection of RSV infections has been described using monoclonal and polyclonal antibodies in immunofluorescence techniques (DIF, IFA), virus neutralisation assays and ELISA or RT-PCR assays (Rothbarth, 1988; Van Milaan, 1994; Coggins, 1998).

Closely related to hRSV are the bovine (bRSV), ovine (oRSV) and caprine RSV (oRSV), from which bRSV has been studied most extensively. Based on sequence homology with hRSV, the ruminant RSVs are classified within the *Pneumovirus* genus, subfamily *Pneumovirinae* (Collins, 1996). Diagnosis of ruminant RSV infection and subtyping is based on the combined use of serology, antigen detection, virus isolation and RT-PCR assays (Uttenthal, 1996;Valarcher, 1999; Oberst, 1993; Vilcek, 1994).

Several analyses on the molecular organisation of bRSV have been performed using human and bovine antisera, monoclonal antibodies and cDNA probes. These analyses revealed that the protein composition of hRSV and bRSV are very similar and the genomic organisation of bRSV resembles that of hRSV. For both bRSV and hRSV, the G and F proteins represent the major neutralisation and protective antigens. The G protein is highly variable between the hRSV subtypes and between hRSV and bRSV (53 and 28% respectively) (Prozzi, 1997; Lerch, 1990). The F proteins of hRSV and bRSV strains present comparable structural characteristics and antigenic relatedness. The F protein of bRSV shows 80-81% homology with hRSV, while the two hRSV subtypes share 90% homology in F (Walravens, K. 1990).

Studies based on the use of hRSV and bRSV specific monoclonal antibodies have suggested the existence of different antigenic subtypes of bRSV. Subtypes A, B, and AB are distinguished based on reaction patterns of monoclonal antibodies specific for the G protein (Furze, 1994; Prozzi, 1997; Elvander, 1998). The epidemiology of bRSV is very similar to that of hRSV. Spontaneous infection in young cattle is frequently associated with severe respiratory signs, whereas experimental infection generally results in milder disease with slight pathologic changes (Elvander, 1996).

RSV has also been isolated from naturally infected sheep (oRSV) (LeaMaster, 1983) and goats (cRSV) (Lehmkuhl, 1980). Both strains share 96% nucleotide sequence with the bovine RSV and are antigenically crossreacting. Therefore, these viruses are also classified within the *Pneumovirus* genus.

A distinct member of the subfamily *Pneumovirinae*, genus *Pneumovirus* is the Pneumonia virus of mice (PVM).

PVM is a common pathogen in laboratory animal colonies, particularly those containing atymic mice. The naturally acquired infection is thought to be asymptomatic, though passage of virus in mouse lungs resulted in overt signs of disease ranging from an upper respiratory tract infection to a fatal pneumonia (Richter, 1988: Weir, 1988).

Restricted serological crossreactivity between the nucleocapsid protein (N) and the phosphoprotein (P) of PVM and hRSV has been described but none of the external proteins show cross-reactivity, and the viruses can be distinguished from each other in virus neutralisation assays (Chambers, 1990a; Gimenez, 1984; Ling, 1989a).

The glycoproteins of PVM appear to differ from those of other paramyxoviruses and resemble those of RSV in terms of their pattern of glycosylation. They differ, however, in terms of processing. Unlike RSV, but similar to the other paramyxoviruses, PVM has haemagglutinating activity with murine erythrocytes, for which the G protein appears to be responsible since a monoclonal antibody to this protein inhibits haemagglutination (Ling, 1989b). The genome of PVM resembles that of hRSV, including two nonstructural proteins at its 3'end and a similar genomic organisation (Chambers, 1990a; Chambers, 1990b). The nucleotide sequences of the PVM NS1/NS2 genes are not detectably homologous with those of hRSV (Chambers, 1991). Some proteins of PVM show strong homology with hRSV (N: 60%, and F: 38 to 40%) while G is distinctly different (the amino acid sequence is 31 % longer) (Barr, 1991; Barr, 1994; Chambers, 1992). The PVM P gene, but not that of RSV or APV, has been reported to encode a second ORF, representing a unique PVM protein (Collins, 1996). New PVM isolates are identified by virus isolation, heamagglutination assays, virus neutralisation assay and various immuno-fluorescence techniques.

Table with addedum: Amino acid homology between the different viruses within the genus *Pneumovirus* of the subfamily *Pneumovirinae*.

Gene	hRSV's	bRSV's	oRSV v. hRSV	bRSV v. hRSV	bRSV v. oRSV	PVM vs. hRSV
NS1	87			68-69	89	*
NS2	92			83-84	87	*
N	96		93			60
P	-		81			
M	-		89			
F	89			80-81		38-40
G	53	88-100	21-29	38-41	60-62	*
M2	92		94			41
SH	76		45-50		56	
L	-					

* No detectable sequence homology

The genus *Metapneumovirus*

Avian pneumoviruses (APV) has been identified as the aetiological agent of turkey rhinotracheitis (McDougall, 1986; Collins, 1988) and is therefore often referred to as turkey rhinotracheitis virus (TRTV). The disease is an upper respiratory tract infection of turkeys, resulting in high morbidity and variable, but often high, mortality. In turkey hens, the virus can also induce substantial reductions in egg production. The same virus can also infect chickens, but in this species, the role of the virus as a primary pathogen is less clearly defined, although it is commonly associated with swollen head syndrome (SHS) in breeder chicken (Cook, 2000).

The virions are pleiomorphic, though mainly spherical, with sizes ranging from 70 to 600 nm and the nucleocapsid, containing the linear, non-segmented, negative-sense RNA genome, shows helical symmetry (Collins, 1986; Giraud, 1986). This morphology resembles that of members of the family *Paramyxoviridae*. Analyses of the APV-encoded proteins and RNAs suggested that of the two subfamilies of this family (*Paramyxovirinae* and *Pneumovirinae*), APV most closely resembled the *Pneumovirinae* (Collins, 1988; Ling, 1988; Cavanagh, 1988).

APV has no non-structural proteins (NS1 and NS2) and the gene order (3'-N-P-M-F-M2-SH-G-L-5') is different from that of mammalian pneumoviruses such as RSV. APV has therefore recently been classified as the type species for the new genus *Metapneumovirus* (Pringle, 1999).

Differences in neutralisation patterns, ELISA and reactivity with monoclonal antibodies have revealed the existence of different antigenic types of APV. Nucleotide sequencing of the G gene led to the definition of two virus subtypes (A and B), which share only 38% amino acid homology (Collins, 1993; Juhasz, 1994). An APV isolated from Colorado, USA (Cook, 1999), was shown to cross-neutralize poorly with subtype A and B viruses and based on sequence information was designated to a novel subtype, C (Seal, 1998; Seal 2000). Two non-A/non-B APVs were isolated in France, and were shown to be antigenically distinct from subtypes A, B and C. Based on amino acid sequences of the F, L and G genes, these viruses were classified again as a novel subtype, D (Bayon-Auboyer, 2000).

Diagnosis of APV infection can be achieved by virus isolation in chicken or turkey tracheal organ cultures (TOCs) or in Vero cell cultures. A cytopathic effect (CPE) is generally observed after one or two additional passages. This CPE is characterised by scattered focal areas of cell rounding leading to synctyial formation (Buys, 1989). A number of serology assays, including IF and virus neutralisation assays have been developed. Detection of antibodies to APV by ELISA is the most commonly used method (O'Loan, 1989; Gulati, 2000). Recently, the polymerase chain reaction (PCR) has been used to diagnose APV infections. Swabs taken from the oesophagus can be used as the starting material (Bayon-Auboyer, 1999; Shin, 2000)

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Addendum

Summary of characterisation of MPV in relation to the other paramyxoviruses.

MPV essentially does not have haemagglutinating activity.
For the genes examined, the newly discovered virus displayed less than 20% amino acid sequence with other non-pneumovirus paramyxoviruses.
Gene constellation: no NS1/NS2 at the 3'end of the genome in MPV.
Genomic organisation: different position of genes between M and L.
Amino acid sequence homologies found with RSV's is low: M: 37%
N: 20-22%
P: 25-27%
F: 32-37%
L: 30-36%

APV is only found in birds/poultry, while we isolated our virus from mammals, in particular humans.
Absence of NS1/NS2 proteins at 3'end , just like APV
Absence of G protein between M and F, just like APV
Amino acid sequence homologies found with APV's is low (but higher than with RSV): M: 75-87%
N: 40-52%
P: 55-67%
F: 66-80%
L: 53-56%

Reason for tentative classification of the virus in the subfamily *Pneumovirinae* (in stead of *Paramyxovirinae*): sequence homology with the *Pneumovirinae*, lack of haemaglutination activity.
Reason for tentative classification of the virus in the genus *Metapneumovirinae*: higher percentage sequence homology with APV then with RSV, apparent lack of NS1 and NS2 genes at the 3' end of the viral genome and probably the same gene order as the APVs. Our PCR and sequence analyses revealed that the G gene is not located between M and F genes (similar to APV and different from RSV), but

further examination of the genomic sequence is required to determine the gene constellation and the gene order between F and L genes. It is possible that equivalents of eg NS1 and NS2 genes of MPV are found elsewhere in the viral genome.

Claims (100) 1

1. An isolated essentially mammalian negative-sense single stranded RNA virus (MPV) belonging to the sub-familie *Pneumovirinae* of the family *Paramyxoviridae* and identifiable as phylogenetically corresponding to the genus *Metapneumovirus*.

2. A virus according to claim 1 comprising a gene order distinct from that of the *Pneumoviruses* within the sub-familie *Pneumovirinae* of the family *Paramyxoviridae*.

3. A virus according to claim 1 or 2 isolatable from humans.

4. A virus according to anyone of claims 1 to 3 comprising an aetiological agent of respiratory tract illness.

5. A virus according to anyone of claims 1 to 4 comprising a nucleic acid or functional fragment phylogenetically corresponding to a sequence shown in figure 6a, 6b, 6c.

6. A virus according to anyone of claims 1 to 5 comprising an MPV isolate deposited as I-2614 at January 19, 2001 with CNCM, Institute Pasteur, Paris or a virus isolate phylogenetically corresponding therewith.

7. A virus according to claim 6 isolatable from a human with respiratory tract illness.

8. An isolated or recombinant nucleic acid or virus-specific functional fragment thereof obtainable from a virus according to anyone of claims 1 to 7.

9. A vector comprising a nucleic acid according to claim 8.

10. A host cell comprising a nucleic acid according to claim 8 or a vector according to claim 9.

11. A proteinaceous molecule or MPV-specific functional fragment thereof encoded by a nucleic acid according to claim 8.

12. An antigen comprising a proteinaceous molecule or MPV-specific functional fragment thereof according to claim 11.

13. An antibody specifically directed against an antigen according to claim 12.

14. A method for identifying a viral isolate as an MPV comprising reacting said viral isolate or a component thereof with an antibody according to claim 13.

15. A method for identifying a viral isolate as an MPV comprising reacting said viral isolate or a component thereof with a nucleic acid according to claim 8.

16. A method according to claim 14 or 15 wherein said MPV comprises a human MPV.

17. A viral isolate identifiable with a method according to anyone of claims 14 to 16 as a mammalian negative-sense single stranded RNA virus within the sub-familie *Pneumovirinae* of the family *Paramyxoviridae* and identifiable as phylogenetically corresponding to the genus *Metapneumovirus*.

18. A method for virologically diagnosing an MPV infection of a mammal comprising determining in a sample of said mammal the presence of a viral isolate or component thereof by reacting said sample with a nucleic acid according to claim 8 or an antibody according to claim 13.

19. A method for serologically diagnosing an MPV infection of a mammal comprising determining in a sample of said mammal the presence of an antibody specially directed against an MPV or component thereof by reacting said sample with a proteinaceous molecule or fragment thereof according to claim 11 or an antigen according to claim 12.

20. A diagnostic kit for diagnosing an MPV infection comprising a virus according to anyone of claims 1 to 7, a nucleic acid according to claim 8, a proteinaceous molecule or fragment thereof according to claim 11, an antigen according to claim 12 and/or an antibody according to claim 13.

21. Use of a virus according to any one claims 1 to 7, a nucleic acid according to claim 8, a vector according to claim 9, a host cell according to claim 10, a proteinaceous molecule or fragment thereof according to claim 11, an antigen according to claim 12, or an antibody according to claim 13 for the production of a pharmaceutical composition.

22. Use according to claim 22 for the production of a pharmaceutical composition for the treatment or prevention of an MPV infection.

23. Use according to claim 22 or 23 for the production of a pharmaceutical composition for the treatment or prevention of respiratory tract illnesses.

24. A pharmaceutical composition comprising a virus according to any one claims 1 to 7, a nucleic acid according to claim 8, a vector according to claim 9, a host cell according to claim 10, a proteinaceous molecule or fragment thereof according to claim 11, an antigen according to claim 12, or an antibody according to claim 13.

25. A method for the treatment or prevention of an MPV infection comprising providing an individual with a pharmaceutical composition according to claim 24.

26. A method for the treatment or prevention of a respiratory illness comprising providing an individual with a pharmaceutical composition according to claim 24.

27. A method according to claim 25 or 26 wherein said individual comprises a human.

28. A method to obtain an antiviral agent useful in the treatment of respiratory tract illness comprising establishing a cell culture or experimental animal comprising a virus according to any one of claims 1 to 7, treating said culture or animal with an candidate antiviral agent, and determining the effect of said agent on said virus or its infection of said culture or animal.

29. An antiviral agent obtainable according to the method of claim 28.

30. Use of an antiviral agent according to claim 29 for the preparation of a pharmaceutical composition.

31. Use according to claim 30 for the preparation of a pharmaceutical composition for the treatment of respiratory tract illness.

32. Use according to claim 30 or 31 for the preparation of a pharmaceutical composition for the treatment of an MPV infection.

33. A pharmaceutical composition comprising an antiviral agent according to claim 29.

34. A method for the treatment or prevention of an MPV infection comprising providing an individual with a pharmaceutical composition according to claim 33.

35. A method for the treatment or prevention of a respiratory illness comprising providing an individual with a pharmaceutical composition according to claim 33.

36. A method according to claim 34 or 35 wherein said individual comprises a human.

Abstract

The invention relates to the field of virology. The invention provides an isolated essentially mammalian negative-sense single stranded RNA virus (MPV) within the sub-familie *Pneumovirinae* of the family *Paramyxoviridae* and identifiable as phylogenetically corresponding to the genus *Metapneumovirus* and components thereof.

TABEL 1

Fig. 1a

M

	00-1	hRSV	bRSV	PMV	APV-A	APV-C	APV-B
00-1	1,00	0,37	0,37	0,37	0,77	0,87	0,75
hRSV	---	1,00	0,91	0,41	0,37	0,37	0,37
bRSV	---	---	1,00	0,42	0,35	0,36	0,35
PMV	---	---	---	1,00	0,37	0,38	0,38
APV-A	---	---	---	---	1,00	0,78	0,89
APV-C	---	---	---	---	---	1,00	0,77
APV-B	---	---	---	---	---	---	1,00

N

	00-1	hRSV	bRSV	PVM	APV-A	APV-C	APV-B
00-1	1,00	0,20	0,22	0,21	0,40	0,52	0,40
hRSV	---	1,00	0,59	0,30	0,18	0,21	0,18
bRSV	---	---	1,00	0,31	0,21	0,23	0,21
PVM	---	---	---	1,00	0,21	0,23	0,21
APVA	---	---	---	---	1,00	0,42	1,00
APVC	---	---	---	---	---	1,00	0,42
APVB	---	---	---	---	---	---	1,00

F

	00-1	hRSV	bRSV	PVM	APV-A	APV-C	APV-B
00-1	1,00	0,32	0,33	0,37	0,67	0,80	0,66
hRSV	---	1,00	0,82	0,40	0,35	0,35	0,35
bRSV	---	---	1,00	0,41	0,34	0,36	0,34
PVM	---	---	---	1,00	0,38	0,38	0,39
APV-A	---	---	---	---	1,00	0,72	0,84
APV-C	---	---	---	---	---	1,00	0,72
APV-B	---	---	---	---	---	---	1,00

P

	00-1	hRSV	bRSV	PMV	APV-A	APV-C
00-1	1,00	0,25	0,26	0,27	0,55	0,67
hRSV	---	1,00	0,81	0,30	0,28	0,26
bRSV	---	---	1,00	0,29	0,28	0,26
PMV	---	---	---	1,00	0,23	0,27
APV-A	---	---	---	---	1,00	0,52
APV-C	---	---	---	---	---	1,00

L8

	00-1	hRSV	bRSV	APV-A
00-1	1,00	0,36	0,35	0,56
hRSV	---	1,00	0,79	0,36
bRSV	---	---	1,00	0,35
APV-A	---	---	---	1,00

L9/10

	00-1	hRSV	bRSV	APV-A
00-1	1,00	0,30	0,30	0,53
hRSV	---	1,00	0,83	0,34
bRSV	---	---	1,00	0,32
APV-A	---	---	---	1,00

Fig. 1b

Table 2

Seroprevalence of hMPV in humans categorised by age group using immunofluorescence and virus neutralisation assays

	Immunofluorescence assays		Virus neutralisation assays		
Age (Years)	N tested	N positive	N tested	N positive	Titre range
< 1	20	5	12	3	16-32
1-2	20	11	13	4	16-32
2-5	20	14	8	3	16-512
5-10	20	20	4	4	32-256
10-20	20	20	4	3	32-128
> 20	20	20	4	3	32-128
8-99[1]	72	72	11	11	16-128

[1]Sero-archeological analysis using sera collected in 1958

Figure 2

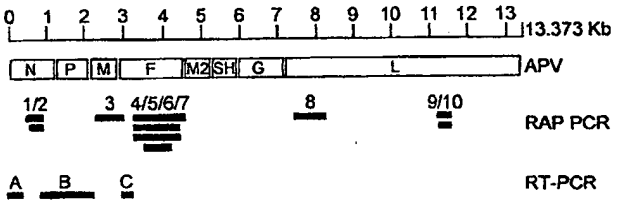

0 1 2 3 4 5 6 7 8 9 10 11 12 13
13.373 Kb
N P M F M2 SH G L
APV
1/2 3 4/5/6/7 8 9/10
RAP PCR
A B C
RT-PCR

FIGURE 3 (3 pages)

Nucleo protein

```
00-1 NP MSLQGIHLSDLSYKHAILKESQYTIKRDVGTTTAVTPSSLQQEITLLCGEILYAKHADYKYAAEIGIQYISTALGSERVQQILRNSGSEVQVVLTRTYSL 10
APV A   ...ES.R....E... ..ED... .R....A...I...E..PQVST...MV.F...T..EP...V.M.......AD.T....K.......G.M.KIVT. 10
APV B   . ....Q..... ..... ..... .....R.VS..... ....T..SH...V.M..V. T..A..T....K. .....A...K.... 10
APV C   ......Q..... ..... ..... .....R.VS. .....T..SH ..V.M..V..T..A..T....K.......A...K.... 10
bRSV    .A.SKVK.N.TFN.DQL.ST.K...Q.ST DNIDIPNYDV.KHLNK ..ML.ITED.NH.FTGL..ML.AMSR..R.DTLK..KDA.YQ.RANGVDVITH 10
hRSV    .A.SKVK.N.TLN.DQL SS.K...Q.ST.DNIDTPNYDV KHLNK...ML.ITED.NH.FTGL..ML.AMSR..R.DTIK..KDA.YH.KANGVDITTY 10
PVM     ...DRLK.N.V.N.DSL.SNCK.SVT.ST.DV.S.SGHAM.KALARTL.MF.LTAFNRCEEV....L..AMSL..RDDSIK...EA.YN.KC.D.QLKDF 10

00-1 NP GKIKNNKGEDLQMLDIHGVEKSWVEEIDKEARKTMATLLKESSGNIPQNQRPSAPDTPIILLCVGALIFTKLASTIEVGLETTVRRANRVLSDALKRYPR 20
APV A   SAEGSVRKREV--.N..D.GVG.ADDVERTT.EA.GAMVR.KV-QLTK..K...L.A.V....I..........V.. ...AI...S......IS.... 19
APV B   ..G..S...E..... R..I..V.... ... SAT.DN..P.....S..A.....I.................A.....N.....F.. 20
APV C   ..G..S...E.. .....R..I..V ..... .SAT.DN. P.... ..S..A.....I..... ..........A.....N.....F.. 20
bRSV    RQDV.G.EMKFEV.TIVSLTSEVQGN.EI.S..SYKKM...M- EVAPEY.HDS..CGM.V...A..VI....AGDRS..TAVI....N..RNEM...KG 19
hRSV    RQDI.G.EMKFEV.TLSSLTSEIQVN.EI.S..SYKKM...M- EVAPEY.HDS..CGM I..IA..VI....AGDRS..TAVI....N..KNEI...KG 19
PVM     TIKLQG.EYKI.V...V.IDAANLADLEIQ..GVV.KE..TG-ARL.D.R.HD...CGV V..IA..VVS...AGDRG..DAVE...LN..KAEKA...N 19

00-1 NP MDIPKIARSFYDLFEQKVYHRSLFIEYGKALGSSSTGSKAESLFVNIFMQAYGAGQTMLRWGVIARSSNNIMLGHVSVQAELKQVTEVYDLVREMGPESG 30
APV A   ....R..K..FE...K...Y.N..........T....RM..... ..............................R..S......K..... 29
APV B   I.................Y..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 30
APV C   I..... .....Y.... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 30
bRSV    LIPKD..N...EV..KYPHYIDV VHF.I.QS.TRG..RV.GI.AGL..N.....V.....L.K.VK.....A.....ME..V...EYAQKL.G.A. 29
hRSV    LIPKD..N...EV..KHPHLIDV VHF.I.QS TRG..RV.GI AGL..N...S. V... ..L.K.VK.....A.....ME..V...EYAQKL.G.A. 29
PVM     .EVKQ..E..... P.P.YIDV .TF L.QS.VRG.  V.G. SGL..N .....V.. ..LL.K.VK.....A.....ME..V...EYAQKQ.G.A. 29

00-1 NP LLHLRQSPKAGLLSLANCPNFASVVLGNASGLGIIGMYRGRVPNTELFSAAESYAKSLKESNKINFSSLGLTDEEKEAAEHFLNVSDDSQNDYE      39
APV A   ..... . .T .. . . ..A .. . .K..A..L .A.. . RT.R.N. ..LAA ....D.R...TSY.GGD.ERSSKF.      39
APV B   ... ..N... . . .. . . . L . . .. A... .R. .... . .....E... ...N...INEEG.....      39
APV C   ......N..... ..... . . . . .L.. ... .. .A... .R. .... .....E.....N...INEEG.....      39
bRSV    FY ILNN...S....TQF  S. . ...A.  M.E...TPR.QD YD. KA .EQ ..NGV..Y.V.D..T..L .IKNQ..PK.N--DVEL      39
hRSV    FY.ILNN...S ...TQF. .S.....A. ..M E...TPR.QD.YD..KA..EQ...NGV. Y.V.D .A..L..IKNQ..PKE.--DVEL      39
PVM     FY.I.NN...S....T.. ..T .... .A. ....S.K.APR R...D  KD..ER..DN.V. Y.A.N..A..R.LISQQ..IV..TPD.DI      39
```

Phospho protein

```
00-1 P MS-FPEGKDILFMGNEAAKLAEAF--------QKSLRKPGHKRSQSIIGEKVNTVSETLELPTISRPAKPTIPSEPKLAWTDKGGATKTEIKQAIKVMDP 91
APV-A ..-.... ..M. S . .M D.Y--------.R.. NTSAG-GR..S. PI..IA.KVP. PLCN.TT.----------..SCI.PNKAPVP..K-- 75
APV-C  .-........L.......A.. .-------- R..K.I..R.T...V.D.II.....V.K....KST.V.T.P.R.N..GE.PDT.RSQTEE.RNEAT. 91
bRSV   .----------.....-------..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 80
hRSV   .EK.APE----.H.ED.NKK.TK.LES----------IKGKF----------ASSKDPKK.DS.ISVNS 45
PVM    .EK.APE----.V.ED.N.K..E.LKHRSFPSE.P.AGIPNTATHVTKYNMPPILRSSFK..SPRVA.NL.E..A.----PTTPPP.PPQN.EEQPKESD 92

00-1 P IEEEESTEKKVLPSSDGKTPAEKKLKPSTNT-----KKKVSFTPNEP---GKYTKLEKDALDLSDNEEEDAE-SSILTFEE--RDTSSLSIEARLESIE 13
APV-A --.I..IYP.LPTAPVATDTYTSTSTE.AKK-----S...K.DNPKV---.......EEG.E....P..DND.K..*.....--K..A.T.....A.. 16
APV-C  EDASRLY.EVFA.T..... .GKETPEKP-----....T.KND.S---.R.....ME..E.....DD...-..V.....--K...A..L.....D 13
bRSV   ..... ..... .....-----..... ...---..... .....-----..... 16
hRSV   .DI.VTK.SPITSGTNIIN.TSEADSTPETKANYPR PL ..KEDLTPSDNPFS..Y.ETIETF---DNN--.EE.SYSY..INDQ.-NDN.T...DR.D 13
PVM    VDI.TMHVC..PDNPEHSKNPCCSDDTD.KKT---R.PM T.VEP EKFV.LGAS.YRETMQTF---AADGYDEE.N.S...TNQEPG.S.V.Q..DR.. 13

00-1 P EKLSMILGLDKTLNIATMSPTAMSDGIRDAMIGVHEELIALIIKEA---KGKAAEM---MESEMXQRSKIGNGSVKLTEKAKELNKIVEDESTSGESEEE 27
APV-A .....M.A . .. . . ... . M  NS.MT..--- D.I. --- K..DT..A...D.....L..Q.S.....S. 25
APV-C ..... ./ . .  . . V I . . .. ---.  ...--- K. AK.K.....G..... 27
bRSV   ..... .. .. . . . . . . . . .----. .... --- . ..X..... 26
hRSV   ....E.. M.H..VV ... .S ..... . I  H.EK RA. LMTNDRLEA.APLRN. SEKMA DTSDE.P.NPTS.K.SDLL..N--------- 23
PVM    ....Y.I...N.IMV.  T . D ...I . .  LM.KSDILTVNDRIVA.EKLKD..CSRADTDDGSACY..DR.RI.D ..SSNA-------- 27

00-1 P EEPKDTQDNSQEDDIY----QLIM 29
APV-A ..SGESESDEE.S...NLDL-.L 28
APV-C ..EE.EEESNPD..L.SLTM-.LIKN 29
bRSV   ..... ..... ..----..... 28
hRSV   ----.SDNDLSL.-------DF. 24
PVM    ---EEAKEDLDV. MGINF- LI 29
```

Matrix protein

```
00-1 matrix MESYLVDTYQGIPYTAAVQVDLIEKDLLPASLTIWFPLFQANTPPAVLLDQLKTLTITTLYAASQNGPILKVNASAQGAAMEVLPKKFEVNATVAKDEYS 10
APV-B       ....II.... V.. ... .. V...NN..K..V.   ..SS..AP..... .S...Q TV.PE..V.Q...T......SA.....S.S.AA.L.... 10
APV-A       ....II.....V.... .. .  ...SN..T..V.  ...SS..AP... .....S...Q.T..PE..V.Q...A......SA.....A.S.A..L.... 10
APV-C       ..........V.......T..V...Q.. R..V.V....T....T...E .  .... ..T... ..............SA...S.D.S.S..L.D.. 10
bRSV        ..T.VNKLHE.ST......YNV..  DD.  ....V.M..SSISADL IKE INVN.LVRQISTLK..S..IMINSRS.VLAQM.S..TIS.N.SL..R. 10
hRSV        ..T.VNKLHE.ST...  .YNVL. .DD  . . .V M .SSV.ADL IKE ASIN.LVKQISTPK..S.R.TINSRS.VLAQM.SN.IIS.N.SL..R. 10
PVM         ..A.. EM.H.V ..   . LN V  HSANI. .V.I M..TSL.KNSVM L.HDV.VICTQISTVH..MI..DL.SSN.GLATM.RQ.LI..II.L.DWG 10

00-1 matrix KLEFDKLTVCEVKTVYLTTMKPYGMVSKFVSSAKSVGKKTHDLIALCDFMDLEKNTPVTIPAFIKSVSIKESESATVEAAISSEADQALTQAKIAPYAGL 20
APV-B        .D.GV....D.RA.  . L........I.TNMNT..R.  .... ...I.M.RGI......Y..A....D ....  ....G.....I...R....... 20
APV-A       R...GT....D.RSI  . L..  ....IMTDVR...R  .........I.I..GV I....Y..A. ..D...........G.....I...R....... 20
APV-C        ..........L.A ..... .... ..N...A.....  ...L....GV......Y..................G.....I...R....... 20
bRSV        ..AY.IT.P..I.ACS..CL.VKN.LTTVKDLTMKTFNP..EI....E.ENIMTSKR.V..T.LR.INV AKDLDSL.NIATT.FKN.I.N...I..... 20
hRSV        ..AY.VT.P..I.ACS..CL.VKS.LTTVKDLTMKTFNP..EI....E.ENIMTSKR.I..TYLRPI V.NKDLNSL.NIATT.FKN.I.N...I..... 20
PVM         NMDYEVPVAFDK.SFCV.IL..KN.LYTVP.ITPTN-RP..E...V.S.HNRVTLKSFN..V..RALY.RQQGLDS..Q....DV.H.I.T.RV..... 19

00-1 matrix IMIMTMNNPKGIFKKLGAGTQVIVELGAYVQAESISKICKTWSHQGTRYVLKSR.                                              25
APV-B       .LL.A........R.............P.. ...LG......N..R...-----I-----L--K----SR                              25
APV-A       .L.................M.......P.. ...LG......N..R.....R..-.GYPK-A.-IC.C-YSQ.K                           27
APV-C       ...................V. ........ .....R..RN...........----------R                                       25
bRSV        VLVI.VTDN..A..YIKPQS.F..D....LEK...YYVTTN.K.TA.KFSI.P----------IED.                                   25
hRSV        VLVI.VTDN..A..YIKPQS.F..D....LEK...YYVTTN.K.TA..FSI.P----------LED                                    25
PVM         TLVINITST..A..L.K..S.ILA...P.LTQV.LHDVIMN.K.T. S.I...SS----------TSG.                                 25
```

Fusion protein

```
00-1 F MSW---KVVIXFSLLI------TPXHGLKESYLEESCSTITEGYLSVLRTGWYTNVFTLEVGDVENLTCADGPS---LIKTELDLTKSALRELRTVSADQ 88
APV-A .DV---RICLLLF.IS------N.SSCIQ.T.N.  . .V.R..K. ....... ..N..I.N.. I..N....---..D...V...N.....K...... 88
APV-B .YL---.LLLIIY.VV------GASGKIQ.T.S  ....V.R..K. .... .... N .I.N...I..N....---..S...S..QN..Q.......... 88
APV-C .. ---...LLLV..A------ .TG..E...  ..Y..V.R  .. .... .................T....---..R. .E.. N..E..K...... 88
bRSV  .ATTAMRMI.SIIFISTYVTHI.LCQNIT.EFYQST. AVSR.  .A... .  S V.I.LSKIQKNV KSTD.KVK ..Q..ERYNN.VV..QSIMQNE 10
hRSV  .ELLIHRLSAI.LT.AINALYL.SSQNIT.EFYQST..AVSR..F.A.. ..  S.I I.LSNIKETK.NGTDTKVK.. Q...KY.N.VT..QLIMQNT 10
PVM   .----IPGR.FLV..VIFNTKPIHPNT T.K.Y.ST  VE.A .K.A.... HMT MSIKLSQINIES KSSN.--- LAH..AIYS..VD....L.SNA 93

00-1 F LAREEQ----------------------IENPRQSRFVLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVRELK 16
APV-A V.K.SR----------------------LSS. RR .   ....  ....  . ..L... ...G .K .....RN ..  .... .........ND.. 16
APV-B ITK.NR----------------------.LSH KK  . ......  .T .    ..L.......G..K ..L..RS ..  ........I.....ND.. 16
APV-C ..K.AR---------------------- MS  KA.. ...       ...      .. ...  . G..A...G  R  ..  .. .  .... ...ND.. 16
bRSV  P.SFSRAKRGIPELIHYTRNSTKKFYGLMGKK KR. L--GFL  IG--S. AS ..VS.VLH. G. NK..   LS..K  VS.S  .S..TSK.LD.. 19
hRSV  P.ANNRARREAPQYMNYTINTTKNLNVS.SKK KR. L--GFL   G--S.IAS I.VS.VLH. G. NK.    LS..K .VS.S  .S..TSK.LD.. 19
PVM   ----------------------------LKSK.KK  L--GLI .LG--.     . L.. VQ... IAL.RD.VRN.   .VS.T  MS. .KV.DD.. 16

00-1 F DFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMTDAELARAVSNMPTSAGQIKLMLENRAMVRRKGFGFLIGVYGSS 26
APV-A E I..K  P. .Q. .N ..I.  I..G.N .     ...   .S... S V.  .  .D..V .INR....S   S. .N. ..  .... I...  DGT 26
APV-B E I..K .P. .Q  ..N ..IP .I..G N. .. .. . ...S ...S.V..  . . ..VK INR... S  .S . N. ... . ...I......GT 26
APV-C ..I .K..P. .R ..  S.    . ..G Y . .. . ... ..... ..... ..V.   .... S  .N .   .     ... I...  ... 26
bRSV  NYID.E.LPQV.NHD.R SNIETVIE.Q K.N.L EIA E..V ... TPL.TYML NS..LSLIND..ITND K.  SS VQI .QQSYSIMSV.KEEV 29
hRSV  NYINNQ.LPIV QQS.R.SNIETVIE.Q.K.S.L EIN E. V...V TPL.TYML.NS..LSLIND..ITND K...SS VQI  QQSYSIMSIIKEEV 29
PVM   N.I. E.LPK..PVS. VH.ITAVIR.Q L K.L.E S E. S...L HTV.SFML  R..TSI GG.AV    KEI .SSK.IM..N.LAI.SS NADT 26

00-1 F VIXMVQLPIFGVIDTPCWIVKAAPSC--SGKKGNYACLLREDQGWYCQNAGSTVYYPNEKDCETRGDHVFCDTAAGINVAEQSXECNINISTTNYPCKVS 36
APV-A .VY  ..  .   E  ..R V  .L --RKE      I .    . T. ...A... KD ..V.D.Y  . .. .. .LEVEQ..Y....SK ..  . 36
APV-B  VY       . .E.  R V  L --RHERES              T .   A   .D ..V D.Y.  . .   .  SEVEQ..H....ST.....  36
APV-C  VYI .           K    L --  D                                E  V S            KE E . R. . .K.. .  36
bRSV  :AYV     Y       KLHTS L TTDN E SDI  T T R   D   VSLF QTET KVQSNR  .  MNSLTLPTDVNL  TD FN K.D. IM 39
hRSV  LAYV     /       KLHTY L TTNI E SDI  T T R   D   VSFF QADT KVQSNR    MNSLTLPSEVSL  TD FNSK.D  IM 39
PVM   LVYVI    L   M  D .VIRSLID --HNIAIN:   A A N   H    D  T.SPT   IHNGYA   LKSLT PVT R.  S MY. . D  I  35

00-1 F TGRHPISMVALSPLGALVACYKGVSCSIGSNKVGIIKQLNKGCSYITNQDADTVTIDNTVYQLSKVEGEQHVIKGRPVSSSFDPVKFPEDQFNVALDQVF 46
APV-A  .   V  . T  .G. S  ES   .   K      G   TH.P.NE  I    .     V   RT   A  VNN N IL  ..  . . .  46
APV-B  . . V.   .T   G. S..E       K          TH P KE  I    I     V   RT   A .VNN.N LL  ..  .. . .  46
APV-C  . . .   .    .    D M  .   K    RF G     S .                . T   K    N   IE  ..  .I      46
bRSV   SKTD  SSVITSI  I S. GKTK TASNKNR    TFSN  D.VS KGV   SVG  L YVN L  KALY  E I:NYY  LV  S.E DASIA  N 49
hRSV   SKTD  SSVITS   I S. GKTK TASNKNR    TFSN  D VS KGV   SVG  L YVN L  KNLYV  E I:NYY  LV  S.E DASIS  N 49
PVM    SKTYV TAV.TTM.C  S  G-N  TVIN DK   KT DD  H  S KGV E OVG    Y   EV KSI VR E LVLKY  LS  D.K D  IRD E 45

00-1 F ESIENSQALVDQSNRILS----SAEKGNTGEII--VIILIAVLGSTMILVSVFIIIKKTKRPTGAP-PELSGVTNNGFIPH-N.                 54
APV-A     DR  D I Y .DL G----AIN SKA IA --A VVLVI  IFFL AVIYICRVR TRIKHDY ATT  DSMAYV----------S              53
APV-B     VDK KD I K  DL D----IEV I I AVL--A TIIV  SKLI VGIAYFVV  R AK SNGY KTT QS M Y S.                     53
APV-C     V K  N I    K I---- I    A  V --  V  VL MLAVVG.G  FVV  P AAPKF -M MN  N K    - -F LLKKKKKKKKK     55
bRSV  AK.NQ L FIRR DEL H--SVDVG .T NVV TTI  V V VILML A.GLLFYC TKST IMLGKDQ   IN LS S----------K              57
hRSV  .K NQ L FIRR DEL H--NVNTG ST NIM TTI  V I VLLSL AIGLLLYC AANT VTLSKDQ   IN IA S----------K              57
PVM   H  NQTRTFFKA EQL DLSENREN NLNKSY LTTLLFVVM III AVIGFILYKVLK----MIRDNK KSKSTP LT----------VLS
```

L polymerase RAP PCR fragment 8

```
00-1 fragment 8 --------TVNVYLPDSYLKGVISFSETNAIGSCLLKRPYLKNDNTAKVAIENPVIEHVRLKNAVNSKMKISDYK-----IVEPVNMQHE 77
APV-A           ME-ISNESV.....V.N...I.D.Y..H..MT.....Q..RALFK.LTISRE.R-----V...LMI.K. 84
bRSV            MDTLIHENST....T.....C..L..Y..DG.....Y.NIISRQK.L...IN..KLSIIQSFVTK.NKGELGLE..TYF.SL 90
hRSV            MDPIINGNSA....T.G.....C..L..YIFNG.....Y.NLISRQ..L...MN..KLNITQSL..K.HKGEIKLE..TYF.SL 90

00-1 fragment 8 IM--KNVHSCEL----TLLKQFLTRSKNISTLKLNMICDWLQLK----------STSDDTSILSFIDVEFI---------- 13
APV-A           LL--.VAAGAR.----KK..KW.G...D..EV..K.VT...K.S---------Q.FGRGK.IDR.Q..NL---------- 13
bRSV            L.TY.SLSTS..ITTT..F.KIIR.AIE..DV.VYA.LNK.G..EKGKVDRC---DDTN.TLSNIVRDNILSVISDNTPSTKKPNNSSCK 17
hRSV            L.TY.SMT.S.QIATTN...KIIR.AIE..DV.VYA.LNK.G..EKDKIKSNNGQDE.NSV.TTI.KDDILSAVKDNQSHLKADKNHSTK 18

00-1 fragment 8 --------------------PSWVSNWFSNWYNLNKLILEFRKEEVIRTGSIL--CRSLGKLVFVVSSYGCIVKSNKSKRVSFFTYNQLL 20
APV-A           --------------------.D.LEH..DS.LI..DV.QSY.CL..SQ.SA..--RK.SLNFF.A...F...II.R..R.IC.C..... 20
bRSV            PDQPIKTTILCKLLSSMSHP.T.LIH..NLYTK..DILTQY.TN.ARNH.Y..IDT.T..EFQ.ILNQ.....YHK.L.KITIT....F. 26
hRSV            QKDTIKTTLLKKLMCSMQHP...LIH..NLYTK..NILTQY.SN..KNH.FT.IDNQT.SGFQ.ILNQ.....YHKEL..ITVT....F. 27

00-1 fragment 8 TWKDVMLSRFNANFCIWVSNSLNENQEGVGLRSNL------Q                                                23
APV-A           ....LA.....L.V....C..SA.D.L....K.VGELLNR                                                24
bRSV            ....IS...L.VCMIT.I..C..TLNKSL...C                                                        30
hRSV            ....IS...L.VCLIT.I..C..TLNKSL.                                                           30
```

L polymerase RAP-PCR fragment 9/10

```
00-1 fragment 9/10 --KLVDKITSDQHIFSPDKIDMLTLGKMLMP--TIKGQKTDQ-----FLNKRENYFHGNNLIESLSAALAXHWCGILTEQC 72
APV-A                -F.S.R..VT.....N..H..LVM...L.L.--.VRSNINNN-----KPAT..F.N...IV.A.TSC..C...TV.ILLT 72
bRSV                 -ICKLNQVIQK..M.L....SLSQYVELFLSNK.L.NSPHISSNLVLVH.MSD..LHKYV----..TN..G..IM.IQLMK 76
hRSV                 DIHKLKQVIQK..M.L....SLTQYVELFLSNK.L.SGSHVNSNLILAH.ISD...NTYI----..TN..G..IL.IQLMK 77

00-1 fragment 9/10 IENNIFKKDWGDGFISDHAFMDFKIFLCVFKTKLLC                                                10
APV-A                T..S..Q.E......T....IN.TW..MS...Y. .HW                                              11
bRSV                 DSKG..E....E.Y.T..M.L.LNV.FDAY..Y.                                                  11
hRSV                 DSKG..E....E.Y.T..M.INL.V.FNAY..Y                                                   11
```

Figure 4

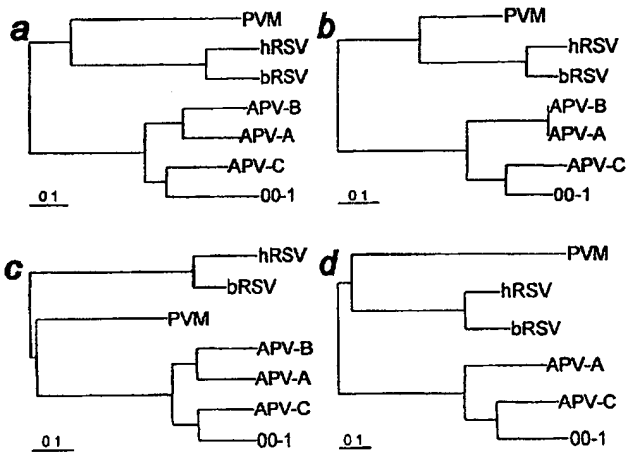

a
PVM
hRSV
bRSV
APV-B
APV-A
APV-C
00-1
0 1
b
PVM
hRSV
bRSV
APV-B
APV-A
APV-C
00-1
0 1
c
hRSV
bRSV
PVM
APV-B
APV-A
APV-C
00-1
0 1
d
PVM
hRSV
bRSV
APV-A
APV-C
00-1
0 1

Figure 5

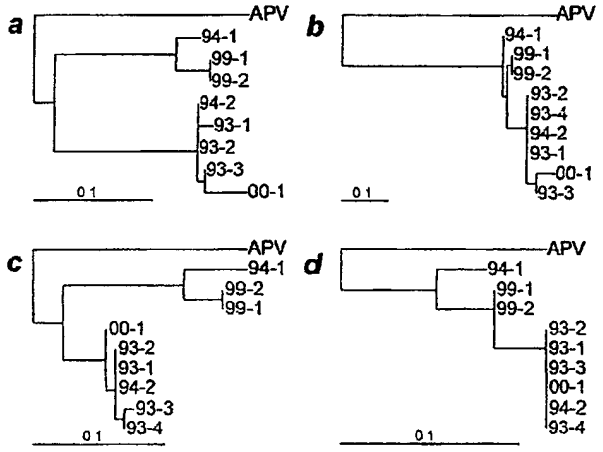

a
APV
94-1
99-1
99-2
94-2
93-1
93-2
93-3
00-1
0 1
b
APV
94-1
99-1
99-2
93-2
93-4
94-2
93-1
00-1
93-3
0 1
c
APV
94-1
99-2
99-1
00-1
93-2
93-1
94-2
93-3
93-4
0 1
d
APV
94-1
99-1
99-2
93-2
93-1
93-3
00-1
94-2
93-4
0 1

Figure 6A part 1

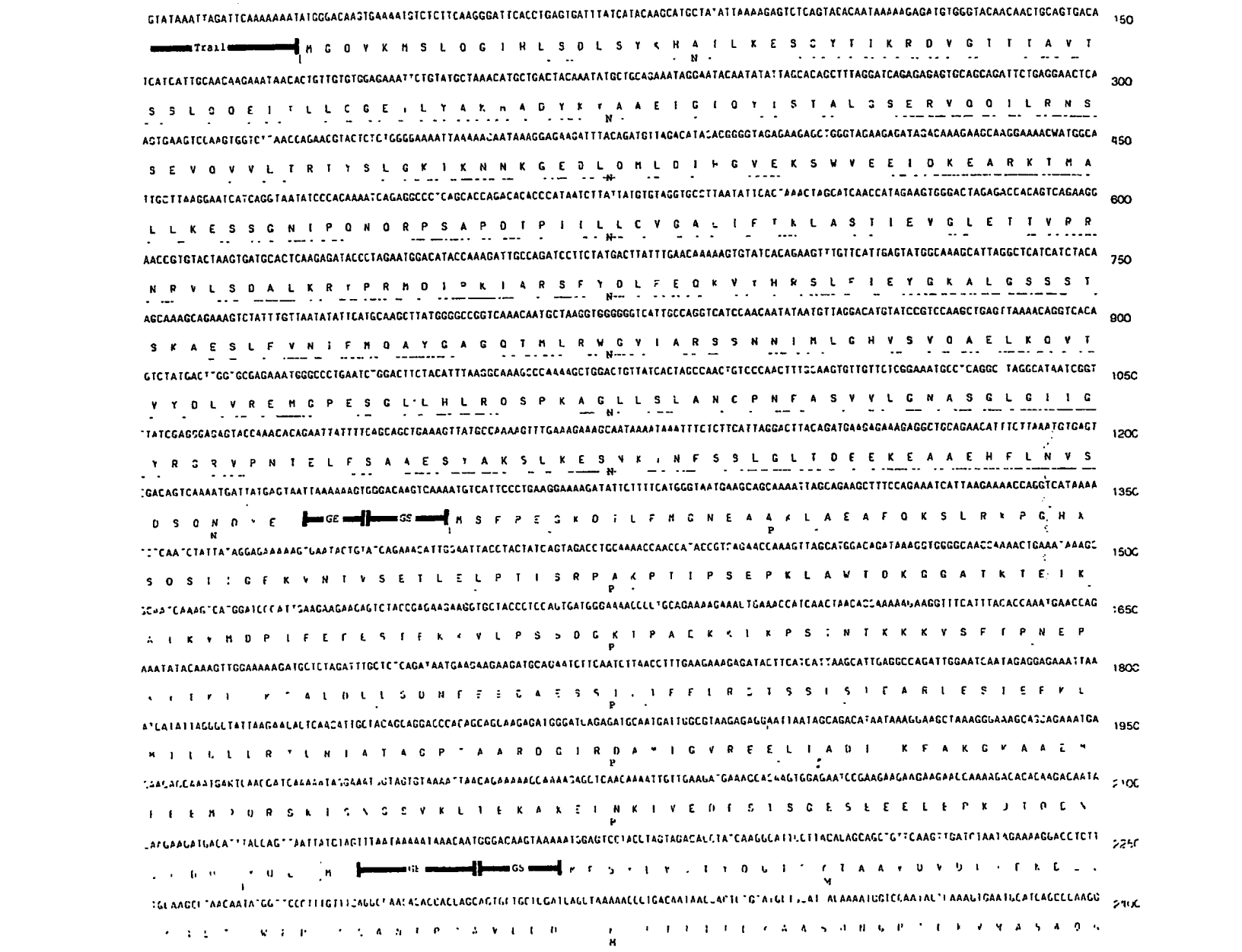


Figure 6A: part 2

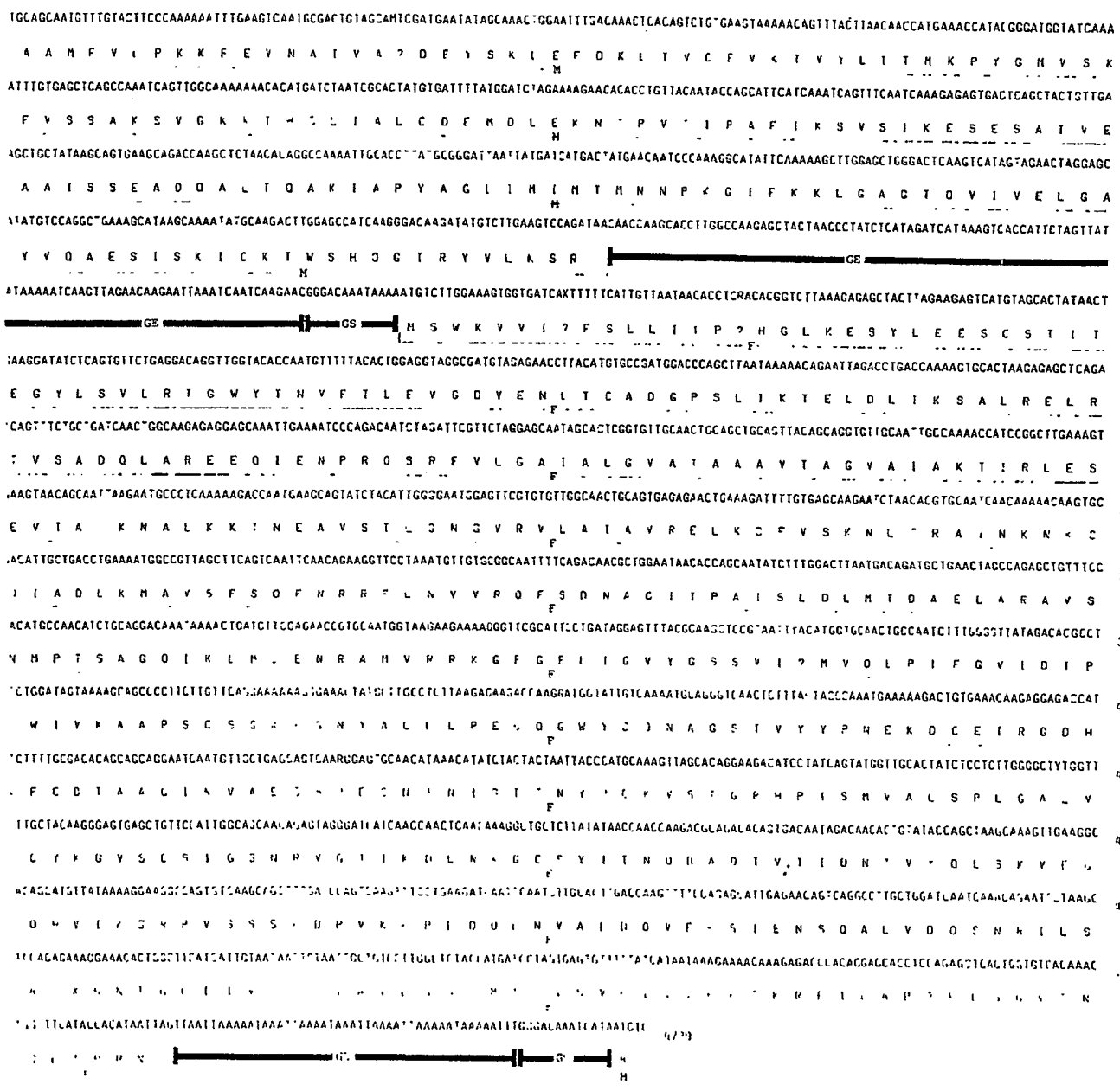
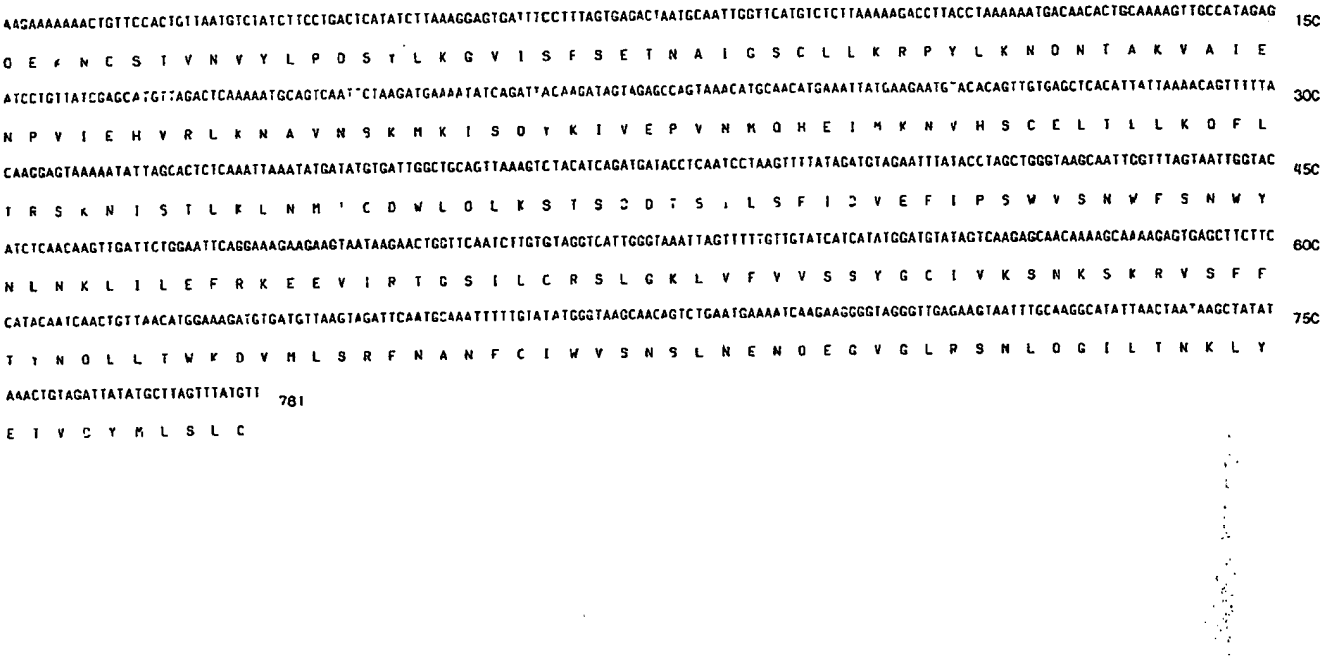


Figure 6B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Q E K N C S T V N V Y L P D S T L K G V I S F S E T N A I G S C L L K R P Y L K N D N T A K V A I E
ATCCTGTTATCGAGCATGTTAGACTCAAAAATGCAGTCAATTCTAAGATGAAAATATCAGATTACAAGATAGTAGAGCCAGTAAACATGCAACATGAAATTATGAAGAATGTACACAGTTGTGAGCTCACATTATTAAAACAGTTTTA 30C
N P V I E H V R L K N A V N S K M K I S D Y K I V E P V N M Q H E I M K N V H S C E L T L L K Q F L
CAAGGAGTAAAAATATTAGCACTCTCAAATTAAATATGATATGTGATTGGCTGCAGTTAAAGTCTACATCAGATGATACCTCAATCCTAAGTTTTATAGATGTAGAATTTATACCTAGCTGGGTAAGCAATTGGTTTAGTAATTGGTAC 45C
T R S K N I S T L K L N M I C D W L Q L K S T S D T S I L S F I D V E F I P S W V S N W F S N W Y
ATCTCAACAAGTTGATTCTGGAATTCAGGAAAGAAGAAGTAATAAGAACTGGTTCAATCTTGTGTAGGTCATTGGGTAAATTAGTTTTGTTGTATCATCATATGGATGTATAGTCAAGAGCAACAAAAGCAAAAGAGTGAGCTTCTTC 60C
N L N K L I L E F R K E E V I R T G S I L C R S L G K L V F V V S S Y G C I V K S N K S K R V S F F
CATACAATCAACTGTTAACATGGAAAGATGTGATGTTAAGTAGATTCAATGCAAATTTTTGTATATGGGTAAGCAACAGTCTGAATGAAAATCAAGAAGGGGTAGGGTTGAGAAGTAATTTGCAAGGCATATTAACTAATAAGCTATAT 75C
T Y N Q L L T W K D V M L S R F N A N F C I W V S N S L N E N Q E G V G L R S N L Q G I L T N K L Y
AAACTGTAGATTATATGCTTAGTTTATGTT 781
E T V C Y M L S L C

Figure 6C

ATAAGCTAGTAGATAAGATAACTTCTGATCAACATATCTTCAGTCCAGACAAAATAGATATGTTAACAC?GGGGAAAATGCTCATGCCCACTATAAAAGGTCAGAAAAC?AGATCAGT?CC?GAACAAGAGAGAGAATTATTTCCATGGGA
K L V O K I Y S D O H I F S P O K I D M L T L G K ? L M P T I K G O K T D O F L N K R E N Y F H G

ATAATCTTATTGAGTCTTTGTCAGCAGCGTTAGCATGTCATTGGTGTGGGATATTAACAGAGCAATGTATAGAAAATAATATTTTCAAGAAAGACTGGGGTGACGGGTTCATATCGGATCATGCTTTTATGGACTTC4AAATATTCCTAT
N N L I E S L S A A L A C H W C G I L T E C C I E N N I F K K D W G D G F I S D H A F M O F K I F L

GTGTCT?AAAAC?AAACTTTTATGTA 327
C V F K T K L L C